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Corrigendum

Vol. III, p. 307. Delete reference in the Bulloch bibliography to obituary notice of Leonard Doncaster (*Proc. Roy. Soc.*, 1921) This was written by William Bateson, not William Bulloch

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THE PHYSIOLOGY OF CAPSULATED STREPTOCOCCI

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(PLATES I AND II)

WORK reported previously (Morison, 1940) indicated that streptococci isolated from human infections were capsulated during a period of their growth cycle and that the presence of a capsule, influenced by certain conditions of cultivation, helped to explain variations in colony form. The arrangement of the individual cocci in the young intact colony was studied and the appearances justified, and indeed, required, the assumption that there was present a capsular material not stainable by the usual methods. This material was revealed by the use of India ink. Capsulated organisms could also be shown to appear in the body fluids of animals dying from virulent infections (fig. 1). This report, like others reviewed at the time, was mainly descriptive, and there has been little consideration of the biological behaviour of the capsule.

Kendall *et al.* (1937) described a complex carbohydrate best isolated from the supernatant broth of 24-hour-old cultures of mucoid or capsulated variants of streptococci of group A. It was not present in significant amounts in cultures of the smooth or non-capsulated variants. It was regarded as capsular material and was a polysaccharide acid composed of equal numbers of N-acetylglucosamine and glucuronic acid units. It was similar to the polysaccharide acid described in bovine vitreous humour and identified as hyaluronic acid (Meyer and Palmer, 1934), and also found in the umbilical cord (Meyer and Palmer, 1936). A similar polysaccharide was later isolated from synovial fluid mucin (Meyer *et al.*, 1939), from a filterable fowl sarcoma (Kabat, 1939) and from the viscous pleural fluid in a case described as a

mesothelioma or endothelioma of the pleura (Meyer and Chaffee, 1940a). Robertson *et al.* (1940) described a mucin from connective tissue as similar to that of synovial fluid. Hyaluronic acid exists, but probably in combination as a sulphuric acid ester, in the cornea and perhaps in the skin (Meyer and Chaffee, 1940b; Claude, 1940; Chain and Duthie, 1940).

Seastone (1939) described a quantitative turbidity reaction given by hyaluronic acid and used it to study some of the factors influencing the liberation of the acid from streptococci of group C. He correlated the loss of capsules, shown by staining, with the appearance of such a substance in the supernatant fluid and considered the loss to be an autolytic process. Meyer *et al.* (1940) appear to have regarded the destruction of the capsule as probably dependent on the same enzyme as splits purified hyaluronic acid with the release of reducing substances. Such an enzyme, a hyaluronidase, was isolated from one strain of streptococci and from pneumococci. Somewhat similar enzymes were also found in *Clostridium welchii* and in ox spleen.

The relationship between hyaluronidase, which releases reducing substances, and mucinase, described as capable of altering the simpler mucins and reducing their viscosity, is discussed by Robertson *et al.* (1940), McClean and Hale (1940) and Hobby *et al.* (1941). It has not been proved conclusively that these two enzymes are completely identical. Again, whether mucinase or hyaluronidase is identical with the Duran-Reynolds spreading factor has not been finally settled (Chain and Duthie, 1939, 1940; Meyer and Chaffee, 1940b; Claude, 1940; Hobby *et al.*, 1941). It would appear that not all preparations showing spreading factor activity contain a comparable amount of hyaluronidase. Many of the extracts tested must consist of a complexity of unidentified enzyme mechanisms and some of these may be more important both in the spreading factor phenomenon and in capsular lysis.

A more direct approach to the phenomenon of capsulation and one more directly related to the morphology of the organisms appears both possible and useful. Such a study must be largely conducted *in vitro* and is a necessary prelude to any appreciation of the possible significance of capsulation in the many outstanding problems of bacterial invasiveness and virulence among streptococci.

TECHNIQUE

Strains. All streptococci used belonged to group A (Lancefield). The greater part of this work was conducted with three strains, one of very high mouse virulence, one isolated from a case of scarlet fever and the third an old stock laboratory strain. Other representative strains were used to confirm the more significant findings.

Capsulated organisms. These were readily obtained by growth, usually for about 3 hours, in Wright's infusion broth with 5-10 per cent. of horse serum and 1 per cent. of glucose added. The inoculum used was equivalent to at least one-hundredth of the volume of medium. It has not been found necessary to replace peptone with neopeptone (Difco) in good quality broth. Washings from blood agar surfaces were very rarely employed. Organisms were deposited with an angle centrifuge and resuspended under the experimental conditions required, usually in fresh broth. In general a 0.9 per cent. solution of NaCl proved unsatisfactory as a suspending medium and the behaviour of the capsules on organisms suspended in it was much less consistent. A more normal environment such as fresh culture medium was preferred as standard. The possibility that fresh growth might occur during manipulations was prevented when necessary by chilling.

Demonstration of capsules. The India ink method of Butt *et al.* (1936) is highly satisfactory. Provided the films are not too thick it consistently demonstrates small differences in the size of the capsules. The staining with methylene blue plainly differentiates non capsulated organisms. The stability of the capsules under the given experimental conditions and the time of their disappearance were studied by examination at frequent intervals and comparison with a suitable control of the same culture. By this rather tedious procedure variations in stability have become very evident. Variations in different cultures even of the same strain make it absolutely necessary to carry a strictly comparative control in every test.

Capsular products. The reaction described by Seastone for the detection of hyaluronic acid depends on the turbidity produced by adding 1 c.c. of 10 per cent. horse serum, buffered at pH 4.2, to 0.4 c.c. of the clear supernatant fluid mixed with 5 c.c. of 0.5 *M* acetate buffer at pH 4.2. The more marked turbidity obtained on adjusting the supernatant fluid of undiluted serum broth cultures to pH 4.2 also depends on the formation and precipitation of an artificial mucin. The reaction cannot be entirely specific, but it serves to indicate the appearance of a constituent of the capsule. Some digest broths and neopeptone are to be avoided. Strong salt solutions inhibit the reaction.

Bacterial multiplication. Many difficulties beset accurate counts of streptococci and multiplication has been estimated only by comparison of the density of suspensions. When necessary the capsules can be removed from organisms at a temperature above that permitting further growth.

Hæmolysin. Titres, determined in doubling dilutions, were only compared in the same experiment and with the same sheep red cell suspension. Where necessary oxidation-reduction potentials were controlled with sodium hydrosulphite.

CAPSULAR SYNTHESIS

The development of capsules varies both with the strain and with the culture medium. Capsules are well marked only during active growth and are best developed in rich media containing serum and with an actively growing and well adapted inoculum. Capsulation has ceased before the period of marked growth decline and as the capsules rapidly disappear there is no reason to believe that capsulation in streptococci represents a preparation for the super-vention of unfavourable conditions, as is suggested by Hoogerheide (1939) for Friedlander's bacillus.

The development of capsules depends somewhat on the state of the inoculum. When organisms which have just lost their capsules are transferred to fresh medium, capsules may sometimes reappear within 15 minutes. If an inoculum from a 36-hour culture, which will long have ceased to produce capsules, is introduced into a fresh medium, a few small capsules may appear after half-an-hour, distributed in an entirely irregular manner on the coccal chains. Even after one hour only a proportion of the multiplying cocci may be capsulated (fig. 2)

Capsules are usually well marked throughout the first 24 hours of growth at 18°-20° C., when growth or anabolic activity as well as katabolic activity or breakdown is slow as compared with what

occurs at 37° C. Indeed, capsulation often appears to be facilitated by growth at temperatures below 37° C. At temperatures just above 37° C. multiplication is slowed, anabolism suffers most interference and the more active breakdown may completely prevent the development of capsules. Capsulation may be considered to depend on the balance of synthesis over destruction.

Especially after repeated subculture in broth without single colony selection some irregularity in the size of capsules and in their stability may be seen (fig. 3). Attempts to fix permanently some examples of such variation within a given strain have failed, and there is evidence of a continual fluctuation in the capsular synthetic mechanisms. This limited variation in capsular development, as well as the variation imposed by differences in the individual suitability of the conditions of cultivation, necessitates some care in comparing the behaviour of different strains.

THE CONSTITUENTS OF THE CAPSULE

There is no information as to how hyaluronic acid, identified by Kendall *et al.* and Seastone as a capsular constituent, is linked to the organisms, or as to whether it exists free in the bacterial membrane. Hyaluronic acid itself is not very soluble in water but its salts are highly soluble. Hyaluronic acid forms simple mucins resembling synovial joint mucin by simple salt linkages through its carboxyl groups with the basic amino-groups of proteins. Such mucins are easily extracted even in weak neutral salt solutions, and unless the protein fractions are very strongly basic they dissociate at a neutral or very weakly alkaline reaction (Meyer, 1938). In the native fluids of the body they apparently occur largely in the dissociated form (Meyer and Chaffee, 1940a). Firmer union of carbohydrate to protein occurs in the sulphuric acid esters such as those found in the cornea and probably in the skin. To bring these into solution, concentrated salt solution, a more alkaline reaction and sometimes a peptising agent such as a concentrated solution of urea are necessary (Meyer and Chaffee, 1940b).

The solubility of the capsule in different salt solutions and in solutions adjusted to different hydrogen ion concentrations has been studied chiefly at 4° C. to minimise enzyme reactions. The capsules are relatively stable. After violent shaking with sand for 2 hours in either broth or neutral 9.0 per cent. NaCl, the capsules are only a little reduced in size, while many of the coccal chains may be disrupted (fig. 4). They are often well preserved after 7 days in 9.0 per cent. neutral NaCl at 4° C. and their stability does not differ from those of organisms suspended in culture medium or glucose (fig. 5). Sometimes, however, especially if they are rather small and beginning to break down, they are less well preserved (fig. 6). This breakdown, and indeed all capsular disintegration, has appeared to be most marked in pure 0.9 per cent. NaCl. Capsules at different periods of their existence may consist of components of varying stability. In any case the complex chemical

CAPSULES OF STREPTOCOCCI



FIG. 1 — Peritoneal exudate of mouse killed 18 hours after inoculation of 10^{-8} c.c. of an overnight broth culture of mouse virulent strain D M Indiana ink. $\times 1250$



FIG. 2 — Irregular development of capsules in a one hour culture from a 30 hour inoculum. Strain Chalk. $\times 1000$

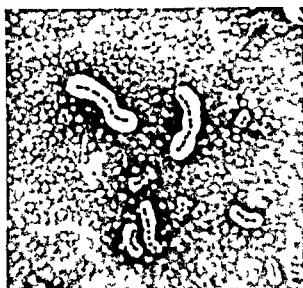


FIG. 3 — A 4 hour culture showing irregularity in size and in capsular breakdown among capsulated organisms. There is evidence that the small clear areas represent incompletely disintegrated capsular material. Strain Chalk. $\times 1000$

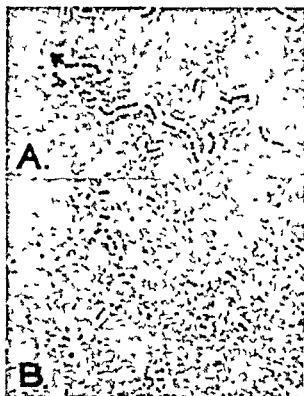


FIG. 4 — A 3 hour culture of capsulated organisms (A) untreated, (B) suspended in 9.0 per cent NaCl and shaken for two hours with sand, capsules only slightly reduced. Strain Cook. $\times 1000$

and probably enzymatic changes leading to capsular disintegration appear to be facilitated at certain stages by relatively low concentrations of NaCl, especially in the absence of other complex organic material. Very concentrated solutions of urea considerably accelerate the disappearance of capsules in suspensions at ice-box temperatures. Hydrogen ion concentration changes between pH 9.0 and pH 4.2 do not markedly facilitate the solution of the capsule, but more acid or alkaline solutions apparently do, and they also interfere with cytological examination. Hyaluronic acid as determined by Seastone's method cannot be extracted independently of some morphological changes in the capsule. These findings would seem to suggest that the capsular material is not free hyaluronic acid and that the union with protein is more stable than in synovial joint mucin. It probably represents a union involving a sulphuric acid ester linkage, or is, as Mudd and Meyer speculated (Meyer, 1938), a very complex linkage between a basic protein, nucleic acid and polysaccharide acid.

Capsular polysaccharide

Seastone's test depends on the formation of an artificial mucin with the hyaluronic acid fraction and is valuable for the detection of the products of capsular breakdown. The opacity produced at pH 4.2 has been correlated with the morphology of the organisms at intervals during growth in 9 capsulated strains. The appearance of a turbidity is always associated with the breakdown of capsules, this breakdown resulting in the release of the reacting substance. No reacting material has been released from washed non-capsulated organisms, either by grinding with sand or by further incubation. Procedures such as those employed by Lancefield (1928) and Fuller (1938) for the isolation of the type and group antigens result in the destruction of any substance reacting in this test. Small amounts of the material may be obtained and purified by allowing capsulated organisms in dense suspensions to disintegrate and then precipitating it from the supernatant fluid with two volumes of alcohol or five volumes of acetone.

Hæmolysin

Rings of hæmolysis appear around the more mucoid colonies on blood agar only when capsules begin to break down. In the strains examined soluble hæmolysin does not appear in serum broth cultures until the capsules show evidence of disintegration. There is then a sudden appearance of thermolabile serum hæmolysin in amounts out of all proportion to any multiplication of the organisms. If the capsulated organisms from 100 c.c. of culture are resuspended in 3.5 c.c. of fluid and the capsules are broken down at 37° C., a potent hæmolysin is obtained. The titre of this is usually at least

4-8 times as great as that of the hæmolysin obtained by allowing the original culture to grow for a further period equal to that occupied in these manipulations. In this experiment the influence of further growth in the concentrated suspension may be somewhat obscure but there is no such difference in titre when suspensions of non-capsulated organisms are similarly treated. Again, if capsulated organisms are exposed in a thin layer of medium in the ice-box for 4 days, any preformed oxygen-sensitive hæmolysin might be destroyed. If now concentrated and non-concentrated suspensions of these still capsulated organisms are exposed to 37° C. for 2 hours, the hæmolysin titres are found to be approximately equal. The hæmolysin now found is probably produced only by fresh growth in both suspensions and none represents that preformed or retained within the capsule. It would be most desirable to eliminate the complication imposed by growth but this has not been possible. Hæmolysin has not been released into non-nutrient suspending fluids. The hæmolysin in the cells is evidently more labile even than that in serum broth and is completely absent when the capsules are destroyed by exposure to 43° C. for one hour. In spite of this, there is some evidence that capsular breakdown at 37° C. releases some hæmolysin independently of further growth and synthesis. However, capsulated organisms are not necessary for hæmolysin production and even true rough variants produce hæmolysin (Dawson *et al.*, 1938). There is no evidence that hæmolysin is produced by the breakdown of capsular constituents but hæmolysin may accumulate within the capsule of the organism.

A hæmolysin bound to the surface of young streptococci and extracted by shaking with inactivated serum was described by Weld (1934, 1935), Schlüter and Schmidt (1936), Hare (1937) and Cotoni and Pochon (1938). The possibility of a relationship between this and the hæmolysin whose release is at least delayed by the presence of capsules has been investigated. No hæmolysin whatever could be extracted, and shaking with glass fragments had little effect on the morphology of the capsulated organisms.

CAPSULAR DISINTEGRATION

A variety of conditions has been investigated in an endeavour to determine the factors responsible for the loss of the capsule and in the hope of controlling them. Differences in growth activity and in the optimal requirements for capsulation exist between different strains. Even in the same strain at different periods these factors cannot be completely controlled. This necessitates great caution before advancing conclusions, especially those implying a relationship between capsular stability *in vitro* and animal virulence. Nevertheless, some results can be reported in fairly general terms.

CAPSULES OF STREPTOCOCCI

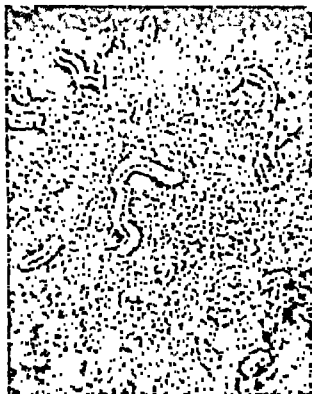


FIG. 5.—Uniformly capsulated organisms from a 3-hour culture resuspended in 9.0 per cent. NaCl for 6 days at 4° C. Strain Cook. $\times 1000$.

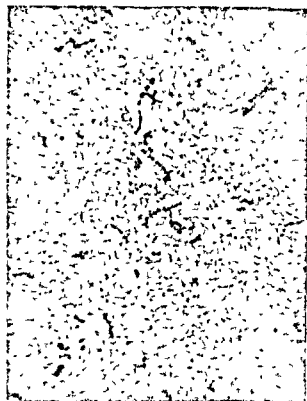


FIG. 6.—Irregularly capsulated organisms of same culture as fig. 3 suspended in 0.9 per cent. NaCl for 24 hours at 4° C. Only some of the larger capsules are preserved. Strain Chalk. $\times 1000$

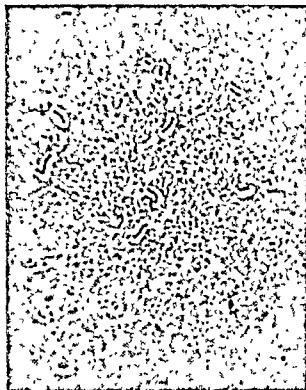


FIG. 7.—Capsules on formalised and washed organisms after 4 hours at 37° C. in heated enzyme extract. In the active extract capsules have usually almost entirely disappeared by this time. Strain Cook. $\times 1000$.

Temperature

Uniform exposure of a culture to 56° C for 12 minutes in the medium in which it has grown or in fresh broth will usually kill the organisms. Some preparations lose only a fraction of their capsular material. In others, many cocci appear naked and others, even in the same chain, may still be capsulated. Not infrequently all the organisms may be deprived of their capsules. If capsules are partially preserved, further exposure at 56° or 37° C results in further loss of capsular material, and though the loss may appear less rapid, all attempts to inhibit capsular breakdown by the use of heat at this or any higher temperature have failed. A temperature of 43° C usually results in destruction of capsules within one hour. Concentrated suspensions of capsulated cocci at 37° C lose the greater part of their capsules in 60-90 minutes. In spite of further growth, cultures of well capsulated organisms have often lost their capsules after a further two hours. In some very mucoid strains capsules are produced even when growth is less active and these may appear to persist longer. At 18°-20° C, suspensions of capsulated organisms which have passed their period of active growth are usually largely devoid of capsules after 12-18 hours. At temperatures of 0°-4° C there is little detectable capsular disintegration for several days, and marked change is not often seen until the seventh or even the fourteenth day. Experimentally, at all temperatures, capsulated organisms in very dense suspensions are if anything less stable than those in dilute suspensions.

The influence of temperature is not that expected for a process involving a simple solution in a non saturated solvent. The reaction to the rise of temperature may be modified by physical changes, such as that from a hydrophobe gel to a hydrophilic sol. An enzymic alteration of the capsular substance would best explain both the influence of temperature and the normal breakdown of the capsule during growth with the liberation of hyaluronic acid.

Hydrogen ion concentration

In broth suspensions of capsulated organisms the influence of the hydrogen ion concentration alone and independent of growth cannot be studied within the temperature range of active growth. However, it can easily be shown that such changes as may occur in the medium during growth are alone unable to influence significantly the breakdown of the capsule. In saline suspensions of group C streptococci Seastone believed that disintegration was increased both above and below pH 6.0.

Oxidising and reducing agents

The presence of organic material renders the measurement of the effective concentration of some of these agents difficult. A

wide range of concentrations has been employed. Hydrogen peroxide and iodine appear to accelerate greatly capsular breakdown even in the cold, and so far no lower concentrations have been found effectively to inhibit capsular lysis. The production of oxidising conditions in bacterial cultures occurs much too late to explain capsular disintegration. Strong reducing potentials maintained with cysteine and with sodium hydrosulphite failed to influence the rate of capsular breakdown as compared with that of similar untreated organisms exposed to temperatures of 43° and 56° C.

Formalin. Contact with formalin in concentrations between 0.5 and 1.0 per cent. will often permit good preservation of streptococcal capsules for 15-20 hours at 37° C. Organisms exposed to even 1.0 per cent. formalin in the ice chest for 24 hours and then thoroughly washed are less stable and may show considerable reduction of capsules within 5 hours at 37° C. Strong reducing potentials maintained with thioglycollic acid in broth resuspensions of these washed organisms had very little influence on this. It has not been determined if this phenomenon depends on a partial reactivation of a capsule-splitting enzyme.

Dubos (1937 *a, b, c*) has reported an interesting series of observations on the intracellular enzymes of the pneumococcus responsible for its disintegration, for the loss of Gram-positive staining and for the loss of its type-specific antigenicity. Later (Dubos, 1938) he showed that low but lethal concentrations of formalin might even accelerate this autolytic process and that pneumococci exposed to higher concentrations were stable. However, when washed free of formalin these pneumococci underwent the change from the Gram-positive to the Gram-negative state. This partial reactivation of the autolytic enzyme, or enzymes, of the pneumococcus is probably more complete than that of the streptococcal capsule-splitting enzyme. The influence of hydrogen ions, iodine and other factors on the pneumococcal enzyme mechanisms is also more apparent.

Other substances. Mercuric chloride (1:1000), gentian violet (0.2 per cent.) and carbon monoxide (to saturation) failed to inhibit or appreciably retard capsular destruction. In lethal concentrations ethyl alcohol accelerates capsular breakdown even in the cold. Bile in a dilution of 1:5 causes almost complete disintegration of the capsules in 30 minutes at 18° C. Sulphanilamide in no way influences the breakdown of the already formed capsule.

In summary, the rate of loss of the capsule is markedly influenced by variations in temperature. The process is not one of simple solution and is not dictated by any change in reaction or in oxidation-reduction potential which could occur during growth. It is suggested that it may best be regarded as depending on an enzyme reaction. Owing to the stability of the enzyme and the relative rapidity with which it may destroy the capsule it has

proved impracticable to prepare entirely stable suspensions of organisms retaining their capsules. This contrasts with conditions among pneumococci, where methods similar to those here employed for streptococci show that capsules are preserved on heat killed organisms as used for the preparation of type sera. This difference in stability may be of some importance.

THE CAPSULE DESTROYING ENZYME

If the supernatant fluids from cultures of organisms which have lost their capsules are readjusted to a suitable hydrogen ion concentration fresh growth of organisms with well defined capsules can be obtained. The factor producing the destruction of capsules cannot be present in effective concentrations as an extra cellular product. Multiplication of a factor resembling a bacteriophage but limited to destruction of capsules may also be eliminated.

A difficulty confronting further study has been to obtain and preserve the substrate—the unaltered capsular material—and to destroy such capsular destructive mechanisms as are normally associated with it. The use of formalin to 1 per cent provides relatively stable and intact capsular material. By careful controls it seems possible to demonstrate some enzyme like activity in the supernatant fluid obtained when capsulated organisms are re-suspended in a greatly reduced volume and their capsules broken down by exposure to 37° C for 2-3 hours. This lytic activity occurs in spite of the large amounts of the end products of such a reaction which are released along with the enzyme into such concentrated suspensions.

Experiment Well capsulated organisms from a 3 hour growth were treated with formalin in a concentration of 1 per cent. After washing three times in broth the capsules were only slightly reduced in size. Fresh capsulated organisms from 100 c.c. of culture were re-suspended in 3 c.c. of broth for 3 hours at 37° C. Their capsules disintegrated. The supernatant fluid was adjusted to neutrality. Half of this was exposed to 56° C for 2 hours and constituted a control. Another control was fresh infusion broth. Heavy suspensions of the washed formalised organisms were added to each and at intervals samples were studied for evidence of capsular disintegration. Tests were carried out at 43°, 37° and 20° C and in all of eight essentially similar experiments consistent results were obtained. With the unheated material there was a marked acceleration of capsular disintegration as compared with the control (fig. 7). The active unheated supernatant fluid usually contained much irregular mucoid material which had separated during the breakdown of the capsules at 37° C. The filaments and masses of this material were first further broken down, after which the capsules of the added formalised organisms disappeared.

This experiment suggests that small amounts of a thermolabile agent capable of breaking down the capsule are released during capsular destruction. No evidence is available as to the identity

of this substance with hyaluronidase isolated from one strain of streptococci and from pneumococci by Meyer *et al.* (1940). Hyaluronidase has not been available and its influence on the morphology of capsulated streptococci is unknown. It is of interest, however, that Meyer *et al.* (1941) have failed to isolate hyaluronidase from 13 out of 14 strains of streptococci. It seems possible that the mechanism of capsular lysis is of considerably greater complexity.

IMMUNOLOGICAL PROBLEMS

No progress has been made with the immuno-chemistry of the capsule. It seems likely that if the capsular material is to act as an efficient antigen it must be presented as a stable protein-containing complex. Organisms killed as suggested by Loewenthal (1938), even if partially capsulated when injected, will undergo further disintegration. Formalin-treated organisms possess somewhat more stable capsules. The type antigen may be a surface antigen but the type reactions are best given by organisms devoid of capsules. It is possible that fractions resembling the type-specific protein M of Lancefield, the labile antigen of Mudd *et al.* (1938) and the antigenic fraction of Hirst and Lancefield (1939) may contribute to the capsular complex. It is doubtful if the cultures employed or the methods so far used could be expected to preserve the capsular material intact. This failure may be of importance when the low protective potency of streptococcal antisera is compared with that of specific pneumococcal antisera. However, even if preserved intact, the lack of "foreignness" of the capsular carbohydrate fraction may limit its practical utility.

The preparation of organisms either by heat or by formalin and with capsules demonstrably well retained, at least at the time of injection, is somewhat difficult and tedious. Such material must often be prepared afresh for practically each injection. As yet only a few rabbits have received a satisfactory series of injections. Other rabbits have been injected with the unstable mucin resembling that of synovial fluid and formed by acid linkage of hyaluronic acid to serum, and also with organisms devoid of capsules. No precipitin reactions with hyaluronic acid and no agglutination or capsule-swelling reactions with capsulated organisms have been demonstrated. Kendall *et al.* explained the inability of sera prepared against mucoid strains to react with hyaluronic acid as due to the normal presence of the acid in the mammalian body. Meyer *et al.* (1939) obtained no reactions using Loewenthal's method of preparing antigens and found synovial fluid to be inactive as an antigen.

More interesting than the serum reactions are speculations on the possible cellular reactions. If the body cells, especially the mononuclear cells, were to become progressively sensitised to the

whole streptococcus as the result perhaps of repeated streptococcal infections, the presence of relatively large amounts of the specific polysaccharide in certain tissue elements might give some significance and localisation to certain types of cellular reaction. The polysaccharide acid, which might then act as an allergen, is associated with primitive myxomatous cells and their derivatives, these line joint spaces and the serous sacs and they persist in loose interstitial tissue. Thus some basis for a localisation of such lesions as are attributed to streptococcal allergy might be suggested. Such a distribution is not very dissimilar to that of the lesions in rheumatic fever.

SUMMARY

The behaviour of the capsules of group A streptococci under the influence of a variety of physical and chemical agents has been studied.

The capsule is not markedly influenced by physiological variations of hydrogen ion concentration or by concentrated salt solutions. A substance behaving like hyaluronic acid is a chemically linked constituent but the capsule itself is of much greater complexity.

The capsule is rapidly destroyed by heat, and this change, believed to depend on enzyme autolysis, introduces great difficulties into the preparation of stable suspensions of capsulated organisms. This, and the previously described identity of the capsular carbohydrate with that normally present in the mammalian body, may have important implications in immunity and in the production of allergic reactions.

The enzyme like mechanism destroying the capsule is influenced somewhat by formalin but not usefully by a great number of other agents. The enzyme like factor has been separated from cells and its activity demonstrated, but it has not been purified.

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THE CAPSULATION OF STREPTOCOCCI AND ITS RELATION TO DIFFUSION FACTOR (HYALURONIDASE)

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A SUBSTANCE which causes an immediate and dramatic increase in the permeability of the tissues can be obtained from the most diverse sources for example, from mammalian testicle (McCLean 1930 1931, Hoffman and Duran Reynals, 1931), from filtrates and extracts of staphylococci and streptococci (Duran Reynals 1933) from organisms of the gas gangrene group and virulent pneumococci (McCLean 1936) from extracts of malignant tissues (Duran Reynals and Stewart 1931, Boyland and McCLean 1935) from snake and spider venoms (Duran Reynals, 1939) and from leeches (Claude, 1937). The increase in dermal permeability is shown by the rapid disappearance of the bleb produced by the intracutaneous injection of these extracts and by the rapid spread through a large area of skin of any suitable coloured indicator that may be injected together with them. This diffusion is so striking that it may be compared with the different behaviour of a drop of water when placed upon glazed and upon blotting paper. The mechanism whereby this effect is produced was not known until Chain and Duthie (1939) reported that purified preparations of testicular diffusing factor exhibit a remarkable mucolytic activity characterised by a rapid fall in the viscosity of the muco protein of synovial fluid and vitreous humour and the liberation therefrom of reducing substances. This observation suggested that the mucolytic and diffusing activities might be due to the same factor and that the spread in the tissues might be due to the action of the enzyme on the mucin like interfibrillar substance of the dermis. These authors have since published (1940) an investigation into the characters of this enzyme derived from testis and other sources. They reported good agreement in quantitative comparisons of the viscosity reducing activity of enzyme preparations from many sources with their activity as spreading factor in rabbit skin, they succeeded in isolating from rabbit skin a substance closely resembling hyaluronic acid which was acted upon by the enzyme. It had previously been shown by Meyer and Palmer (1936) that the muco protein of vitreous humour and umbilical cord contained a muco polysaccharide which they had designated by the name hyaluronic acid. It seems reasonable therefore to call the enzyme which hydrolyses this substance "hyaluronidase". While this work was in progress Meyer *et al* (1940) reported the hydrolysis of hyaluronic acid by certain bacterial enzymes and Robertson *et al* (1940) described an enzyme derived from culture filtrates of *C. uelchi* and other organisms which hydrolyses synovial fluid, neither of these groups of workers had at that time connected the mucolytic activity of their preparations with the effect on the permeability of the tissues.

McCLean and Hale (1940 *a* and *b*) confirmed the association between diffusion in the dermis and mucolytic activity exhibited by extracts from

various sources. They also reported that the activity of the bacterial enzymes could be completely inhibited by appropriate antisera. They later studied the behaviour of these enzymes and of certain artificially prepared compounds which reduce the viscosity of hyaluronic acid (McCLean and Hale, 1941) and found that the inclusion of potassium hyaluronate in the culture medium of *Cl. welchii* resulted in a considerable increase in the production of hyaluronidase by this organism. This "adaptive" enzyme effect is of considerable interest in relation to the metabolism of all those organisms which produce hyaluronidase and in the pathology of the diseases which they cause. Gas gangrene infections are characterised by extremely rapid spread through the tissues; it appears likely that as soon as *Cl. welchii* starts to proliferate in the tissues the hyaluronic acid there is attacked. Thus the organism is able to use this metabolite and, at the same time, by destroying the viscosity of this material, to increase the permeability of the tissues and render more of the specific substrate accessible. In addition, the presence of hyaluronic acid causes the organism to produce increased amounts of the enzyme; thus a vicious circle is set up which promotes the rapid extension of the infection. Duran-Reynals (1933) has correlated the invasiveness of strains of staphylococcus and streptococcus with the yield of diffusing factor, which may act in a similar manner in infections by these organisms.

It is of interest to determine the part played by hyaluronidase in the virulence and local invasiveness of infections due to organisms that produce this enzyme. In view of the technical difficulties of infection experiments with *Cl. welchii*, it was decided to make a preliminary study of streptococcal hyaluronidase and its relation to the capsulation of these organisms. Bordet (1897) first reported that virulent streptococci develop capsules in the body of the infected animal and resist phagocytosis, the non-capsulated organisms being phagocytosed. Hare (1931) showed that virulent streptococci in young culture resisted phagocytosis by human blood whereas organisms from old cultures were readily phagocytosed. Seastone (1934) correlated the resistance of young cultures to phagocytosis with the presence of a capsule which disappears in older cultures. Ward and Lyons (1935) concluded that, although resistance to phagocytosis is associated with the presence of capsules, this structural difference between virulent and avirulent strains cannot be the sole factor in determining resistance to phagocytosis since certain non-capsulated virulent variants also resisted phagocytosis and one attenuated capsulated variant was readily phagocytosed.

Kendall *et al.* (1937) isolated from the "mucoid" (capsulated) phase of various types of group A streptococci a serologically inactive polysaccharide which appeared to be identical with that isolated by Meyer and Palmer (1936) from vitreous humour and from Wharton's jelly of the umbilical cord. This polysaccharide, like those from the vitreous humour and umbilical cord, was hydrolysed by the autolytic enzyme of the pneumococcus which is now known to be hyaluronidase (Meyer *et al.*, 1937) and which diffuses in the tissues and appears to be strictly specific for hyaluronic acid. Seastone (1939 *a* and *b*) concluded that the virulence of several variants obtained from a group C streptococcus isolated from a guinea-pig epidemic depended upon the possession of capsules which were composed of a serologically inactive polysaccharide similar to, if not identical with, that isolated by Kendall *et al.* from group A organisms. He correlated the disappearance of capsules in ageing cultures with a diminution in the yield of this polysaccharide from the bacterial growth, but was unable to confirm the findings of Pradhan (1937) that young cultures of invasive strains produce both capsules and diffusing factor. In Seastone's opinion the invasiveness of group C

streptococci is associated with the presence of capsules composed of this polysaccharide, which he regarded as a "virulence factor". Thus he does not distinguish between the property of local invasiveness and that of virulence or killing power, but it appears that these two attributes of the organism may vary independently and may depend upon separate factors. It has been suggested that the virulence of streptococci may be associated with the possession of capsules but it is known that other strains which have been described as invasive (Duran Reynals, 1933) secrete an enzyme which increases the permeability of the tissues by its action on mucoprotein.

It therefore seemed desirable to study the production by streptococci of capsules and of this enzyme and to investigate their relation to each other and the part they play in determining either the virulence or invasiveness of the organism. Some of the observations to be described here have already been briefly reported in a preliminary communication (McClean, 1941).

METHODS

Most of the strains of *Streptococcus pyogenes* employed were obtained from the National Collection of Type Cultures and were of known Lancefield group and serological type. Four untyped strains originally isolated from cases of erysipelas (Eagles, 1926) were obtained from the same source. Group B strains, freshly isolated from bovine mastitis, and group C strains from infections in the horse were kindly supplied by Mr S. J. Edwards and Mr K. C. Sellers of the Agricultural Research Council Field Station, Compton. Two "mucoid" group C strains were obtained through the courtesy of Dr E. W. Todd of the L. C. C. Antitoxin Laboratories.

Cultures were stored in the cold in blood broth. Capsule production was observed after 2 hours' incubation in neo-peptone infusion broth to which 20 per cent of serum and 0.1 per cent of glucose had been added. When the supply of neo-peptone failed it was found that Evans's peptone could be used with equivalent results. All the strains were also cultivated on plates containing medium similarly enriched with glucose and serum in order to observe colonial type and capsule production on solid media.

The composition of both broth and solid media was varied in certain experiments by the inclusion of hyaluronic acid or hyaluronidase in order to determine the influence of these substances upon colony formation, capsule production and the formation of hyaluronidase by the organisms.

In the earlier experiments capsules were examined with the aid of Hiss's stain, but the India ink method was found to be superior and was used exclusively in most of the observations. The organisms were counter-stained with Loeffler's methylene blue.

Diffusing activity in the tissues was tested by measuring the area of spread of intracutaneous injections of dilutions in isotonic hemoglobin solution of culture filtrates or centrifuged culture supernatant fluids according to the method described by McClean and Hale (1941).

Hyaluronidase activity was titrated either by viscosimetry (Madinaveitia and Quibell, 1940; McClean and Hale, 1941) or by determining the highest dilution of the supernatant which would digest a substrate of the mucopolysaccharide to the point when this substrate in the presence of serum protein would no longer produce the typical mucin clot on the addition of acetic acid. For some reason, not yet completely understood, it has not been possible to titrate streptococcal hyaluronidase accurately.

by the viscosimetric method successfully employed with enzymes derived from testis, *Cl. welchii* and vibriion septique. The rate of the reaction with streptococcal hyaluronidase does not bear the same direct relation to concentration of enzyme as it does with hyaluronidase from other sources. This experimental difficulty is under investigation. It was known, however (Seastone, 1939b), that the muco-polysaccharide when mixed with serum-protein produces a typical mucin clot in the presence of acid. Experiment showed that, if increasing dilutions of enzyme were incubated with a substrate-serum mixture for 20 minutes in a water-bath at 37° C. and the end-point of destruction of clotting power determined by the addition of a constant amount of acetic acid, the potency of the enzyme could be determined with an error of ± 20 per cent. It was found, moreover, that this in-vitro test gave an end-point comparable with the highest dilution producing increased diffusion in the dermis and there is good correlation between the two tests, thus affording additional evidence that the action on tissue permeability is directly due to hyaluronidase. The time taken to destroy the clotting power of the substrate is not directly proportional to concentration of enzyme, but if the time of the reaction is kept constant the highest effective dilution of enzyme is proportional to the potency of the original sample.

The muco-polysaccharide substrate was prepared from Wharton's jelly of the umbilical cord by the method of Meyer and Palmer. The serum used in the preparation of the serum-substrate mixture should be tested before and after incubation for 20 minutes, to ensure that it does not itself destroy the clotting power of the substrate; samples of serum are occasionally encountered which themselves contain an enzyme which attacks the muco-polysaccharide.

EXPERIMENTAL RESULTS

Behaviour of strains freshly revived from stock cultures

Cultures in broth enriched with serum were examined after 2 and 20 hours' incubation for the presence of capsules and the supernatant fluids were tested for the presence of diffusing factor. The results, together with those obtained after animal passage, are shown in table I.

Two of the four scarlatinal strains (types 1-4) produced capsules, one produced diffusing factor but no capsules and one produced neither. All the other group A strains of known type produced capsules but no diffusing factor after 2 hours' incubation. The four erysipelas strains when first revived showed neither capsules nor diffusing factor. It is only fair to state that these erysipelas strains had been maintained in artificial culture for many years without animal passage.

Four group B strains (untyped) freshly isolated from cases of bovine mastitis failed to develop capsules, although two of these strains ("Mary" and "Tulip 2nd") showed slight but definite halos both in young broth cultures and at 20 hours on serum-enriched plates. These halos did not, however, resemble the typical capsules developed by certain group A and C strains, and, unlike these capsules, they were not susceptible to the action of hyaluronidase, as will be described later. The group B strains all produced moderate

amounts of diffusing factor or hyaluronidase. Quite recently Dr E. W Todd has supplied me with a group B strain (strain 090,

TABLE I

Capsule formation, diffusing factor and hyaluronidase production of freshly received and animal passaged strains of streptococci

Source of strain	Serological type (Griffith)	Before passage			After passage		
		Capsules	Diffusion in the dermis (m d d)	Hyaluronidase	Capsules	Diffusion in the dermis (m d d)	Hyaluronidase
Group A strains							
Scarlatina	1	+	>0.2 ml	10 ⁻²	+	>0.2 ml	0
"	2	—	>0.2 "				
"	3	+	>0.2 "				
"	4	—	10 ⁻² "				
Unknown	8	+	>0.2 "	0	+++	>0.2 ml	0
"	9	+	>0.2 "				
"	10	+	>0.2 "				
"	12	+	>0.2 "				
Erysipelas—							
(N C T C 2366)	Unknown	—	>0.2 "		—	10 ⁻²	10 ⁻²
(N C T C 2616)	"	—	>0.2 "		—	10 ⁻³	10 ⁻²
(N C T C 2619)	"	—	>0.2 "		—	10 ⁻³	10 ⁻²
(N C T C 2367)	"	—	>0.2 "				
Group B strains							
Bovine Mastitis— (" Mary ")	Unknown	Slight halo	10 ⁻² ml	10 ⁻²			
(" Filbert ")	"	—	10 ⁻² "	10 ⁻²			
(" Tulip 2nd ")	"	Slight halo	10 ⁻² "	10 ⁻²			
W2	"	—	10 ⁻² "	10 ⁻²			
Group C strains							
Bovine Mastitis— (N C T C 5983)	Unknown	—	10 ⁻¹ ml				
Unknown	7	—	10 ⁻² "	10 ⁻²	—	10 ⁻⁵	10 ⁻⁵
"	20	—	10 ⁻² "	10 ⁻²	—	10 ⁻⁴	10 ⁻³
"	21	—	10 ⁻² "	10 ⁻²	—	10 ⁻⁵	10 ⁻⁴
Loewenthal M	Unknown	+++	>0.2 ml	0			
" O	"	++	>0.2 "	0			
Foal septicemia	"	+	>0.2 "	0			
Mare 1	"	—	trace	trace			
" 2	"	+	>0.2 "	0			
" 3	"	++	>0.2 "	0			

Diffusion in the dermis: minimal diffusing dose (m d d) = highest dilution of culture supernatant producing increased spread of at least 20 per cent

Hyaluronidase: the figure shown represents the highest dilution of culture supernatant that completely destroyed the clotting power of mucin on the addition of acetic acid. 0 = no destruction of clotting power

N C T C = National Collection of Type Cultures

type 1a) which develops well marked capsules in serum glucose broth. These capsules persist up to at least twenty-four hours'

incubation and are not destroyed by hyaluronidase from any source, thus indicating that group B capsules, when they occur, are not composed of hyaluronic acid. Moreover, this strain itself produces hyaluronidase in high titre. It is not included in table I.

Four group C strains, including one untyped non-hæmolytic strain originally derived from a case of bovine mastitis, all failed to develop capsules but produced moderate amounts of diffusing factor. Two group C "mucoid" strains showed very large capsules and no hyaluronidase or diffusing factor after 2 hours' incubation. Of four strains of equine origin, two showed no capsules and one of these produced traces of hyaluronidase; the other two strains developed definite capsules on a large proportion of the chains and produced no hyaluronidase.

It will be noticed that none of the strains examined produced both capsules and diffusing factor at the same time. Those strains that develop capsules show them after 2 hours' incubation but they have disappeared at 20 hours. The diffusing factor appears after 2 hours but there is a further increase at 20 hours. The supernatant fluids showing diffusion in the skin were tested viscosimetrically for hyaluronidase activity but, as already explained, could not be accurately titrated by this method. They caused a definite fall in the viscosity of the polysaccharide in 30 minutes. The results of the test for the destruction of the clotting power of mucin are shown in table I.

The effect of serial passage through mice

Three representative capsulated group A strains and three group C strains which produced diffusing factor were injected intraperitoneally into mice and recovered from the heart blood after eighteen hours. This passage was repeated four times and resulted in a considerable increase in the virulence of the strains.

The observations on capsules and diffusing factor production were repeated after mouse passage. There was no change in the incidence of capsules but they were more marked and there was approximately a hundredfold increase in the production of diffusing factor by the group C strains. There was also a considerable increase in hyaluronidase activity, measured by the test for the destruction of clotting power and by diffusion in the skin. It was also noticed on centrifuging the cultures that the capsulated strains did not pack down tightly but that the non-capsulated strains did so and were not disturbed when the supernatant fluid was decanted.

Three of the four erysipelas strains were serially passaged through three mice and re-examined for capsule formation and hyaluronidase production. The fourth strain failed to establish an infection and could not be recovered. As will be seen in table I, after this animal passage all three strains showed moderate diffusing

activity and hyaluronidase production and no capsule formation. Erysipelas is an infection with a typical spreading character and it seems likely that hyaluronidase plays a part in determining the nature of this disease. It was unfortunate that no strains freshly isolated from cases of erysipelas were available for examination. Our observation on the diffusing activity of erysipelas strains confirms that of Duran-Reynals (1933), who reported that strains from this source were among the most active examined by him. He showed that the diffusing factor elaborated by invasive organisms passes into the bloodstream, causing a general increase of tissue permeability which may enhance local infections elsewhere.

The action of hyaluronidase on streptococcal capsules

One quarter of a ml of a 2 hour culture of a representative capsulated strain was mixed with an equal volume of purified testicular hyaluronidase and, after one minute, examined for capsules. These had completely disappeared. A control sample mixed with the testicular extract inactivated by heat still showed typical capsules. This observation was repeated with all the capsulated strains by adding a hyaluronidase containing supernatant from one of the group C cultures, with the same result. The capsules of all the 'mucoid' group C strains were also destroyed by hyaluronidase from various sources. Thus it appears that the capsules of both group A and group C streptococci are destroyed by hyaluronidase derived not only from testis and *Cl. uelckii* but also from other streptococci. This confirms the reports of Kendal *et al* and Seastone that the capsules of both groups are composed of hyaluronic acid. The halos and capsules already noticed in the group B strains were not affected by hyaluronidase.

The following observations were made upon the 20 hour culture supernatant of a strain which had shown capsules after 2 hours' incubation. 0.25 ml of this supernatant was mixed with an equal volume of heated and unheated streptococcal and testicular hyaluronidase with the results shown in table II. In control tubes

TABLE II

The precipitation of hyaluronic acid from the supernatant fluid of a 20 hour culture of a capsulated strain of streptococcus

Culture of supernatant fluid (strain type J)	Enzyme or control fluid added	Precipitation with acetic acid
0.25 ml	0.25 ml streptococcal hyaluronidase	—
0.25 "	0.25 " uninoculated broth	+++
0.25 "	0.1 " testis hyaluronidase	—
0.25 "	0.1 " heated testis hyaluronidase	+++
0.5 " uninoculated broth	0.1 " testis hyaluronidase	—
0.5 "	0.1 " heated testis hyaluronidase	+

uninoculated broth was added to the supernatant instead of hyaluronidase and hyaluronidase to the uninoculated broth.

It will be seen that after the addition of active hyaluronidase to this culture supernatant no precipitate developed on the addition of acetic acid; the addition of uninoculated broth or inactivated enzyme did not prevent the appearance of a definite precipitate. The supposition that this precipitate consisted of hyaluronic acid is supported by the fact that it fails to appear after the addition of hyaluronidase and also because the actual addition of 2 drops of a 0.25 per cent. solution of hyaluronic acid to the uninoculated broth produces an exactly similar precipitate when acetic acid is added; the addition of 4 drops of hyaluronic acid solution results in a typical mucin clot in the presence of acetic acid. Table II also shows that, in the presence of heat-inactivated hyaluronidase, uninoculated broth gives a very slight precipitate in the presence of acetic acid; this cannot, however, be confused with the other precipitates referred to above.

It appears, then, that ageing cultures which have been capsulated secrete hyaluronic acid which can be detected in the medium. This is additional evidence that these strains do not produce significant amounts of hyaluronidase in young culture, since this enzyme, if present, would destroy the substrate. The change in the environment of the organisms, produced by their multiplication, which results in the loss of capsules and the liberation of capsular substance, is of considerable interest and requires further investigation.

The results of adding hyaluronic acid or hyaluronidase to the culture medium

The six mouse-passaged strains were each cultivated in a basal medium of 2.5 per cent. Evans's peptone infusion broth, alone and with the addition of 0.1 per cent. of glucose or 1 per cent. of a solution containing hyaluronic acid. Tubes containing glucose or hyaluronic acid were also used with and without the addition of 20 per cent. of serum. The cultures were observed for the appearance of capsules and the production of diffusing factor; hyaluronidase estimations were also made by the mucin clot destruction test.

Only one group A strain (type 9) produced definite capsules in the absence of serum and that only in the tube containing glucose, but they all showed slight halos. All the group A strains developed capsules in the tubes containing serum but the inclusion of hyaluronic acid in the medium appeared to reduce the number of capsulated chains and the amount of capsule. None of the group C strains showed any capsules or any suggestion of halo.

In order to economise in the number of animals used, only two representative strains were examined in all the different media for the presence of diffusing factor. No 20-hour culture of the

capsulated strain in any medium showed significantly increased diffusion in the dermis at a dilution of 1 : 10. The following results were obtained with the various cultures of the non-capsulated strain.

Basal medium alone : diffusion in a dilution of 1 : 1000

Basal medium+glucose : diffusion in a dilution of 1 : 100

Basal medium+hyaluronic acid : diffusion in a dilution of 1 : 1,000,000

Basal medium+serum and glucose : diffusion in a dilution of 1 : 1000

Basal medium+serum+hyaluronic acid : diffusion in a dilution of 1 : 100,000.

The inclusion of hyaluronic acid in the medium increases the production of diffusion factor a thousandfold but the addition of serum does not cause any further increase.

All the cultures of all six strains were tested for hyaluronidase by the in-vitro test for the destruction of the capacity of hyaluronic acid to form a mucin clot. Two-hour cultures of the capsulated strains showed no evidence of hyaluronidase; tests on 20-hour cultures of the same strain suggested that there might be a trace of hyaluronidase in the undiluted broth but nothing in a dilution of 1 : 10. Titrations of hyaluronidase in the three non-capsulated strains gave very consistent results which may be summarised as follows.

Basal medium alone : detectable hyaluronidase in a dilution of 1 : 100

Basal medium+glucose : detectable hyaluronidase in a dilution of 1 : 100

Basal medium+hyaluronic acid : detectable hyaluronidase in a dilution of 1 : 10,000

Basal medium+serum and glucose : detectable hyaluronidase in a dilution of 1 : 100

Basal medium+serum+hyaluronic acid : detectable hyaluronidase in a dilution of 1 : 10,000.

Thus the test confirmed the result of the skin diffusion test and extended it to the other two non-capsulated strains. The experiment indicates that the addition of hyaluronic acid to the culture medium slightly inhibits capsule production in the capsulated strains, but fails to promote the formation of significant amounts of hyaluronidase by these strains. On the other hand, this substance causes marked increase of the hyaluronidase produced by non-capsulated strains.

A similar experiment was made on the influence of the inclusion of hyaluronic acid in the medium on the same strains prior to their passage through mice. The group A type 4 strain, which had already been found to produce hyaluronidase and not to develop capsules,

was examined at the same time. This strain, as well as the other non-capsulated strains, all responded to the inclusion of hyaluronic acid in the medium by a hundredfold increase in hyaluronidase. Two of the three capsulated strains were found to produce a trace of hyaluronidase at 20 hours, after the disappearance of their capsules, and these also responded to hyaluronic acid in the medium by increased production of the enzyme. The fact that capsulated strains, which produce no hyaluronidase in young cultures, do produce a trace of this enzyme after 20 hours' incubation provides a possible explanation for the loss of capsules after the first few hours' cultivation in liquid medium. This trace of enzyme cannot be detected at dilutions higher than 1 : 2, either by the clot test or by diffusion in the skin.

One representative capsulated and one non-capsulated strain after mouse passage were incubated in plates composed of a basal medium of Evans's peptone infusion agar with the inclusion of glucose or hyaluronic acid, both with and without 20 per cent. of serum. In addition, sheep red blood cells were added to plates containing glucose and serum or hyaluronic acid and serum.

The capsulated strain developed typical "mucoid" colonies on the plates containing serum but not in its absence. The organisms in the "mucoid" colonies showed typical capsules even after 20 hours' incubation. A few capsules only could be seen in the absence of serum. This strain, which had caused considerable hæmolysis in the blood broth tube, caused no β hæmolysis on the plates containing red blood cells, either in the presence of glucose or hyaluronic acid. The non-capsulated strain produced no "mucoid" colonies or capsules in any of these media. This strain, however, produced marked zones of β hæmolysis.

The influence of hyaluronic acid and hyaluronidase on hæmolysin formation

A further experiment was performed with the three capsulated strains and another non-capsulated strain to determine the conditions influencing hæmolysin production. The experiment was similar to the last in that glucose or hyaluronic acid was included in the medium, but 20 per cent. of serum was added to all the plates. In addition, testicular extract (hyaluronidase) was added to one set of plates.

"Mucoid" colonies were formed by all the capsulated strains. It was noticed that these colonies showed no zone of hæmolysis, whereas in the group A strain (type 9) some non-mucoid colonies developed and these showed a small zone of hæmolysis and no capsules. The addition of testicular hyaluronidase to the medium on which these strains were grown prevented the appearance of "mucoid" colonies and resulted in the development of small matt

colonies without capsules and the production of small zones of β haemolysis. All the plates of the non-capsulated hyaluronidase-producing strain contained small matt colonies with wide zones of haemolysis; this strain was unaffected by the addition of testicular hyaluronidase. This experiment suggested that strains which are capsulated do not produce haemolysis but that as soon as the capsule is lost, either through the action of hyaluronidase or from some other cause, haemolysis is produced.

A further experiment was performed with the three capsulated and three non-capsulated strains in an attempt to determine whether haemolysin formation is inhibited by the presence of capsules. These strains were cultivated in serum glucose broth with the addition of testicular hyaluronidase, which would prevent capsule formation, and heat-inactivated hyaluronidase. The haemolysin in the broth supernatant was titrated after 2 hours' incubation, when capsules were present in the capsulated strains. No significant difference in the titre of haemolysin produced could be detected between the capsulated and non-capsulated strains nor between those cultivated in the presence of hyaluronidase and those without. It appears, therefore, that in liquid media the presence of capsules on young cultures does not exert any inhibitory action on the production of haemolysin.

Capsules persist up to 20 hours on serum plates and appear to be associated with failure to cause β haemolysis, whereas in serum broth the capsules disappear after 3-6 hours and do not inhibit haemolysin production. These facts suggest that some factor is secreted into the broth which promotes capsule destruction and haemolysin production and that this factor cannot operate in a solid medium.

The relation of the capsules of virulent type 1 pneumococcus to the hyaluronidase produced by this organism and by streptococci

While these experiments were in progress similar observations were made on a strain of type 1 pneumococcus known to produce hyaluronidase (McClean, 1936). Unlike the streptococci, this organism produces both capsules and hyaluronidase and the capsules are not destroyed by either pneumococcal or streptococcal hyaluronidase. On the other hand, pneumococcal hyaluronidase destroys the capsules of streptococci in the same way as streptococcal hyaluronidase. Galacturonic acid and an acetyl hexosamine, probably glucosamine, have been obtained from the capsular substance of type 1 pneumococci (Heidelberger *et al.*, 1936) and it is this substance that determines the type-specificity of the organism. Hyaluronic acid, which composes the streptococcal capsules, consists of equimolar proportions of glucuronic acid and N-acetyl hexosamine and is not type- or group-specific. Therefore,

notwithstanding the close chemical similarity between these two capsular substances, only one of them, that containing glucuronic acid, is attacked by hyaluronidase and this is additional evidence of the strict specificity of its action. Like the streptococcus, type 1 pneumococcus responds to the inclusion of hyaluronic acid in the medium by a hundredfold increase in the production of hyaluronidase.

DISCUSSION

Some strains of *Streptococcus pyogenes* develop capsules in young culture; others produce hyaluronidase and diffusing factor which, if not identical, are very closely related to each other. The development of capsules and hyaluronidase formation appear to be mutually exclusive; they cannot co-exist in the same culture, since hyaluronidase either prevents the development of capsules or destroys them when they are formed. These observations do not confirm those of Pradhan, from which he concluded that both capsules and spreading factor were elaborated by young cultures of the same strain, that they were both the same substance and were neutralised or destroyed as the culture aged.

Hyaluronidase-producing strains of streptococcus and type 1 pneumococcus responded to the inclusion of hyaluronic acid in the medium by a considerable increase in the formation of hyaluronidase. This response is similar to that already described for *Cl. welchii* (McCLean and Hale, 1941) and its influence on the spread of infection in tissues which contain a mucinous inter-fibrillar structure has already been discussed in the introduction to this paper.

It will be extremely interesting to elucidate the respective parts played in streptococcal infection by capsules and by hyaluronidase. Seastone (1939 *a* and *b*) regards the capsule as a "virulence factor" in the strains examined by him, but he uses the terms virulence and invasiveness as interchangeable. It seems necessary to distinguish clearly between the virulence or killing power of an organism and its capacity to invade the tissues and set up an infection which may be more or less localised. Kendall *et al.* (1937) and Seastone (1939 *a* and *b*) have stated that the capsular substance of both group A and group C streptococci is serologically inactive; if this is so and the capsule is a virulence factor, it seems that it must act in a negative way as a protective envelope to the organism so that it cannot be attacked by the immune mechanism of the host.

Hyaluronidase, on the other hand, increases the permeability of the tissues to organisms and their toxins and it may be significant that those erysipelas strains of streptococci that were examined produced hyaluronidase but no capsules. In a study of the relation between tissue permeability and local immunity Favilli and McClean

(1937) have shown that inhibition of the spread of diffusing factors is associated with the early stages of an inflammatory response in the tissues, they concluded that local immunity, at least in so far as it is manifested in the skin is due to a local reduction of tissue permeability which is itself associated with the earliest stages of inflammation and is not primarily a specific immune response. Menkin (1938) reviewed a series of papers in which he had shown how the inflammatory response is characterised at an extremely early stage by a process of fixation whereby the site of inflammation is walled off from the surrounding tissues. It is obvious that any enzyme elaborated by an infecting agent which tends to prevent this fixation and thus increase the area involved may be important in determining the invasive character of the infection. Such an enzyme may enhance the effect of the fibrinolytic and anti-fibrinogenic substances described by Dennis and Berberian (1934) in *Streptococcus hemolyticus* and associated by them with invasiveness.

The factors which determine the loss of capsules after the first few hours' incubation by strains that are capsulated in young culture are of interest in their bearing on the course of an established streptococcal infection and on the metabolism of the organism. There are indications that this loss of capsule may be associated with the appearance of small amounts of hyaluronidase in the older cultures of these strains. It is interesting that in plates enriched with serum the capsules persist up to 20 hours whereas in liquid media they disappear much sooner.

In an interesting study of the relation between colony formation and the capsulation of hemolytic streptococci Morrison (1940) suggests that the degree of capsulation represents the balance between synthesis and destruction of capsular material and that the breakdown of the capsule may be regarded as an enzyme action. He also noticed the difference in behaviour of pneumococci and streptococci when grown on a cellophane surface, in that the pneumococcal capsule persists after growth has slowed down. Morrison found that mouse virulent strains acquire and retain capsules in the peritoneum of the mouse, but that the degree to which capsulation develops *in vitro* is no sufficient guide to the virulence of a strain. During infection experiments which will be reported later, we also have found that mouse virulent strains are capsulated both in the peritoneum and in the heart blood, but we agree that correlation between capsulation in artificial media and virulence is not satisfactory.

SUMMARY AND CONCLUSIONS

1 Some strains of *Streptococcus pyogenes* develop capsules in young cultures, other strains produce hyaluronidase and diffusing factor, which may be identical.

2 Capsules and hyaluronidase cannot co-exist in the same group A or group C strain, since the capsular substance is destroyed

by this enzyme. The inclusion of hyaluronidase in the medium prevents the appearance of capsules.

3. The capacity to form capsules or hyaluronidase is not confined to members of one Lancefield group. The capsules of both group A and group C strains are composed of the same polysaccharide, hyaluronic acid. All group B strains so far examined produce hyaluronidase and only one of these develops typical capsules; these capsules are not affected by hyaluronidase and are not, therefore, composed of hyaluronic acid.

4. Hyaluronidase-producing strains respond to the inclusion of hyaluronic acid in the medium by a considerable increase in the formation of hyaluronidase. It is considered that this response may be of importance in the extension of infections by streptococci and other organisms that produce this enzyme.

5. Virulent type 1 pneumococci produce hyaluronidase and respond in the same way as streptococci to the inclusion of hyaluronic acid in the medium. Pneumococcal hyaluronidase destroys streptococcal capsular substance, but the capsules of this pneumococcus are not attacked by pneumococcal hyaluronidase or that from other sources.

6. The respective parts that may be played by capsules and by hyaluronidase in streptococcal infection are discussed. A clear distinction between the virulence of an organism and its local invasiveness is stressed.

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CERVICAL ABSCESSSES OF GUINEA-PIGS

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In the spring of 1939 occasional guinea pigs amongst the stock bred at the Medical Research Council's farm laboratories, Mill Hill, developed large swellings in the neck. These were usually unilateral but occasionally bilateral. Autopsies showed that they consisted of chronic abscesses with thick creamy pus or caseous material enclosed within a dense fibrous capsule. It was evident from examinations of early cases that the disease originated as an infection of one of the submaxillary lymphatic glands but in advanced cases the cervical lymph glands were also involved while salivary gland tissue was frequently found embedded within the fibrous capsule. In some guinea pigs retrogression with complete recovery occurred, in others the abscesses broke down and pus was discharged through an external sinus.

It was learnt that an outbreak of the disease had occurred some years previously in Cambridge amongst guinea pigs derived from the Mill Hill stock but that attempts to isolate a causative organism had been unsuccessful. A similar condition was also encountered in 1938 at the Lister Institute (Kheneberger 1939, 1940) and I am informed that within the last few months further cases have occurred at Mill Hill. It is evident, therefore, that the infection is not uncommon and may prove a source of trouble in work necessitating the use of guinea-pigs.

Isolation of causative organism

In smears of pus stained by Gram, Giemsa or dilute carbol-fuchsin, structures were sometimes seen suggestive of a filamentous organism but no indubitable microorganisms were recognisable. Isolation by direct culture was attempted in five cases of the natural disease. A wide range of media was employed in each experiment and in each case the inoculum consisted of an emulsion of pus and abscess capsule made by thorough trituration in broth. Only two of the five attempts proved successful. In the first, small granular colonies developed after 3 days in a cooked meat and broth

* Part of the work was done prior to the outbreak of war at the National Institute for Medical Research, Hampstead.

medium incubated anaerobically ; they resembled mould colonies with a compact brownish centre and a lighter fluffy periphery. In the second, similar colonies developed, again under strictly anaerobic conditions, in glucose broth containing 30 per cent. bovine serum which had by that time been found to support growth better than the cooked meat in broth. Subculture presented no difficulty and after a few passages the organism became facultatively aerobic although retaining its preference for anaerobiosis. Finally surface colonies could be obtained on 30 per cent. serum agar plates incubated either with or without the presence of oxygen.

Isolation by animal inoculation, followed by subsequent culture from the lesions produced, proved much more successful than direct culture. The wide pathogenicity of the organism was not known in the early stages of the investigation but inoculation of either guinea-pigs, rabbits or mice was carried out with each of the five suspensions prepared for culture. In this way, the organism was isolated from all five naturally infected guinea-pigs. It is probable that this result was determined to some extent by the sparsity of bacteria in the chronic abscesses, coupled with the fact that many animals received much larger amounts of emulsion than were seeded into the cultures. Nevertheless, evidence was obtained that animal inoculation provided a more sensitive method for primary isolation than did direct culture. The results of the isolation experiments are summarised in the table.

TABLE

Isolation of organisms by primary culture and by animal inoculation

Source of material	Primary culture	Inoculation			
		Guinea-pigs (subcutaneous)	Mice (subcutaneous or intraperitoneal)	Rabbits (subcutaneous)	Rabbits (intratesticular)
Guinea-pig 1 .	+	+	+
" 2 .	—	+	+	+	...
" 3 .	—	—	+
" 4 .	—	+	+	+	+
" 5 .	+	...	+	+	+
No. of attempts	5	4	5	3	2
No. positive .	2	3	5	3	2

Morphology and cultural characteristics

Stained preparations of early cultures showed a tangled mycelium of gram-negative filaments and gram-positive bacilli, together with an abundance of granular material and irregularly stained bizarre forms. It was at once evident that fixed, stained smears were unsuitable for morphological study and examination of living

preparations under dark ground illumination was adopted as a routine practice. Such examinations indicated that the organism was a strain of *Streptobacillus moniliformis* or, adopting the nomenclature proposed by Parker and Hudson (1926), an anaerobic species of the genus *Haverhillia*.

The most striking feature was the extraordinary pleomorphism. Long and short bacilli often forming a tangled mycelium, large and small spheres, balloon, sausage and club shaped structures, granules of various sizes and degrees of refractility and delicate filaments forming loops and arches might be present, either all together or in any combination. The bacillary elements and spheres were usually loaded with highly refractile granules but were sometimes non granular. True branching undoubtedly occurred. It is not proposed to describe the morphology in greater detail as the descriptions and illustrations in the publications of Strangeways (1933) and Klieneberger (1936) could be equally well applied to the guinea pig organism. Four points, however, demand special mention.

(a) Two types of growth occurred in liquid media and two types of colony developed on serum agar plates. Growth in liquid might be in the form either of small compact granules at the bottom of the tube with perfectly clear supernatant fluid or as a flocculo-membranous deposit with some general turbidity, the former showed all the pleomorphic forms described the latter chiefly bacillary forms. On solid media large and small colonies invariably developed. Large colonies were 1.2 mm in diameter after 3 or 4 days' growth, irregularly round, low convex, with a dull granular surface and a brownish tinge. Small colonies were pin point in size, almost invisible to the naked eye, circular in outline, high convex and colourless, with a smooth shiny surface. Small colonies were composed mainly of bacillary forms and sometimes indeed no other elements could be discovered, whereas the large colonies always showed the full range of pleomorphic elements. Persistent efforts to separate permanently these two types of growth were completely unsuccessful. Subcultures from either large or small colonies invariably gave rise to a mixture of both large and small. Similarly, subcultures from serum broth growths of distinctive type might give rise to either of the two types.

(b) The type of growth in liquid media was largely conditioned by the constituents of the media. For example, parallel cultures in broth containing bovine, horse and rabbit serum showed widely different morphology. The addition of a preparation of catalase from horse liver enhanced growth and favoured the development of bacillary forms. The influence of different constituents of culture media upon morphological variation was emphasised by van Rooyen (1936) in his studies of aerobic strains of *Streptobacillus moniliformis*.

matted together to form a large caseous mass adherent to stomach, liver and spleen. Scattered abscesses occurred in any of the viscera and occasionally the lungs or spleen presented an appearance reminiscent of miliary tuberculosis. Intravenous inoculation resulted in an even more widespread generalisation, but it is interesting that an incubation period of about a week elapsed before the mice looked sick. Of a batch of six mice inoculated in this manner, one which died on the eleventh day was the only animal of any species to show infection of a limb joint. One ankle was very swollen and contained pus. In the outbreaks of disease among human beings and stocks of mice attributed to *Streptobacillus moniliformis* infection, arthritis was one of the most constant features (Levaditi *et al.*, 1925, 1932; Parker and Hudson, 1926; Strangeways, 1933; Mackie *et al.*, 1933; van Rooyen, 1936).

Rabbits. Subcutaneous inoculation of rabbits gave rise to extremely chronic abscesses at the site of injection. The incubation period and initial development of the lesion were identical with those in guinea-pigs, but the abscesses slowly enlarged over a period of 2-3 months before regression ensued, and scarcely ever broke down with sinus formation and discharge of pus. The infection spread slowly along the lymphatics, which could sometimes be felt as thick fibrous cords. From direct extension large abscesses might be formed in the groin and in the belly wall extending to the mid-line, but there was never any sign of spread to remote parts by any other route and internal organs were never affected. Inoculation into the testis produced a more rapid infection, with swelling, induration and congestion within 24-48 hours, followed by the development of a purulent orchitis.

Egg membranes. The inoculation of cultures on the chorio-allantoic membranes of 9- or 10-day chick embryos produced a general opacity and thickening of the membrane with cream coloured or yellowish nodular lesions containing caseous material. Sometimes the embryos were dead when the eggs were opened on the 3rd or 4th day, but it was surprising how extensively the membrane could be involved with the embryo alive and active. In some eggs with living embryos, over half the available membrane surface was covered with a thick warty-looking confluent lesion. Dark ground preparations from such lesions were indistinguishable from those made from cultures.

Attempts to reproduce the natural disease

It has been shown that lesions produced experimentally always involved the tissues at the site of inoculation and the adjacent lymphatic vessels and glands, and that, except in mice, infection never spread to remote parts. Even in mice with extensive

generalisation there was no tendency to involvement of the cervical glands. Naturally infected guinea-pigs on the other hand showed involvement of the submaxillary and cervical glands only. Strangeways found that rats frequently carry *Streptobacillus moniliformis* in the nasopharynx; indeed about 50 per cent. of the rats bred at Mill Hill were shown to be carriers. These facts suggested the possibility that food infected by rats might have been the source of infection in the outbreak of the guinea-pig disease, the organisms gaining entrance through abrasions in the mouth or pharynx. An attempt was therefore made to produce cervical abscesses by feeding guinea-pigs with food to which cultures of the organism had been added.

Two experiments were made, in each of which were used groups of young guinea-pigs rigorously isolated since weaning. In the first experiment two of three animals fed with cultures developed typical cervical abscesses, the three controls remaining quite healthy. In the second experiment, however, in which four guinea-pigs were fed with cultures and three kept as controls all remained well. The outbreak of war prevented further attempts, so that although the result of the first experiment is suggestive, the problem of how natural infection occurs remains unsolved.

Discussion

The morphological and cultural peculiarities of the organism isolated from guinea-pig cervical abscesses leave little doubt that it is closely related to the strains of *Streptobacillus moniliformis* or *Haverhillia multiformis* described by previous workers. These have already been reported as responsible for outbreaks of human disease and for epizootics in stocks of mice. The guinea-pig organism differs from the strains previously described in its demand for strict anaerobiosis on first isolation and its exclusively pyogenic action in all the species for which it has been found to be pathogenic. Unlike them, it does not give rise to a general systemic infection with erythema and especial predilection for the joints. It is probable that the genus *Haverhillia* will be found to embrace many widely distributed bacterial species responsible for a number of infective conditions in different hosts. In routine bacteriological laboratories it is not uncommon to receive specimens of pus which prove sterile by the cultural methods employed. In such cases the possibility of *Haverhillia* infection is worth bearing in mind, and both anaerobic cultures on media containing at least 30 per cent. of serum, and animal inoculation, especially intratesticular inoculation of rabbits, are indicated.

Klieneberger has repeatedly reported the association of filterable pleuropneumonia-like organisms in symbiosis with bacteria,

especially those belonging to the *Streptobacillus moniliformis* group. Many of the morphological appearances seen in dark ground preparations of the guinea-pig organism suggested the presence of such a symbiont and consequently several attempts were made to demonstrate one by Klieneberger's methods. All the attempts were completely unsuccessful, nor could any filterable phase of the organism be demonstrated. Pleuropneumonia-like organisms do undoubtedly occur independently in a number of diseases; an organism of this class was isolated by Klieneberger and Steabben (1937) from the lesions of 17 out of 19 rats suffering from a bronchiectatic condition of the lungs. It is significant that in this disease *Streptobacillus moniliformis*, shown by Strangeways to be carried by a high percentage of rats in the nasopharynx, tended to assume the role of a secondary invader of the bronchiectatic lesions. This suggests that the frequent association of the two microorganisms is fortuitous and not based upon any essential interdependence.

The occurrence of bacillary disintegration with the production of globular bodies, spheres and granules, followed by regeneration of bacilli, discounts the view of Klieneberger (1940) that globular bodies always represent an independent symbiotic organism and lends support to the views of Dienes (1938, 1939), who believes that the globular bodies are derived from bacilli and that the so-called L1 organism is a variant of *Streptobacillus moniliformis*, not an independent symbiont. He found that the L1 organisms after isolation and subculture might revert to the typical mixed form.

Whilst many of the globular bodies are unquestionably living organisms, either symbiont or variant, some of the structures described in the literature as characteristic of *Streptobacillus moniliformis* are composed of lifeless material. The "streaming phenomenon" described above emphasises the need for extreme caution in the interpretation of morphological observations, especially those based on fixed and stained preparations.

It was hoped to find the source of infection of guinea-pigs but this line of investigation was interrupted by the outbreak of war. A plausible hypothesis supported by a certain amount of circumstantial evidence is that guinea-pigs are infected through abrasions in the buccal cavity by eating rat-contaminated grain. Should this hypothesis be substantiated prevention of the disease would be easy.

Summary

1. An organism belonging to the *Streptobacillus moniliformis* group was isolated from five cases of cervical abscess in guinea-pigs. Isolation was accomplished from three of the cases by preliminary inoculation of mice and rabbits when direct culture of abscess material yielded no growth.

2 The organism was essentially pyogenic and was pathogenic for guinea pigs, rats, mice and rabbits, but except in mice its invasive power was very low

3 Morphological observations by dark ground illumination showed that some of the bizarre forms described by previous workers are composed of non living elements. Changes of form indicative of a developmental cycle were observed

4 The development of cervical abscesses followed the feeding of guinea pigs with cultures of the organism in one experiment but an attempt to repeat the experiment failed

Thanks are due to Mr R. E. Glover, who collaborated in the feeding experiments

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STREPTOCOCCUS TOXIN-ANTITOXIN FLOCCULATION AND ITS RELATION TO THE RABBIT SKIN TEST

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SINCE Dyer (1925) described a flocculation between scarlet fever toxin and antitoxin similar to the Ramon flocculation, little progress was reported in this field until the publications of O'Meara (1935) and Rane and Wyman (1937). This was due mainly to the difficulty of obtaining flocculation with anything like regularity in scarlet fever toxin-antitoxin mixtures. By blending with a rapidly flocculating antitoxin (O'Meara) or by the use of a concentrated toxin (Rane and Wyman) it is now possible to determine easily and rapidly the flocculating value of any scarlet fever antitoxin. O'Meara concluded that the flocculating substance present in scarlet fever toxins was a specific streptococcal product which played a part in streptococcal infections and to which an antibody was developed during the course of the human disease. It had many of the characteristics of a true toxin, although direct evidence of its toxicity was lacking, but it was not identical or associated with the erythrogenic toxin. Rane and Wyman, however, claimed that the flocculating substance is at least closely associated with the erythrogenic toxin and that the erythrogenic antibody can be accurately determined by the flocculation reaction. Should this claim be substantiated, the partial replacement of the rabbit skin test by a flocculation test would be a distinct advantage since, in many laboratories, the rabbit test has been found difficult to perform, rather inaccurate and both expensive and time-consuming. The objects of this investigation have been to determine whether such a substitution can be made and whether there is a specific flocculation associated with the neutralisation of the erythrogenic toxin by antitoxin. This has been attempted by comparing the flocculation titres with the routine in-vivo values of samples of antitoxin, both concentrated and unconcentrated, produced in these laboratories. The results on the whole seem to confirm the claim of Rane and Wyman.

METHODS

Before proceeding to the experimental observations it is necessary to give some details of the methods and units employed.

In-vivo method

The method used is the rabbit intradermal test described by Buttle and Lowdon (1935) and referred to below as the skin test. All in-vivo values are determined by direct titration against the laboratory standard serum containing 1300 U.S.A. units of scarlet fever antitoxin per c.c. This value is obtained by direct comparison with the standard for scarlet fever streptococcus antitoxin from the National Institute of Health, Washington, D.C., which is stated to contain 40 units per c.c.

In-vitro method

Direct titration of sera with concentrated toxin

Toxins are obtained by growing hæmolytic streptococci (strain *Streptococcus scarlatineæ*, Dochez NY 5) on the medium described by O'Meara (1934), and are concentrated to about one-fifth of their original volume by ultrafiltration. The ultrafiltration is effected through Berkefeld candles coated with a "Parlodion" membrane. The candles are immersed for ten minutes in an 8 per cent. solution in glacial acetic acid of Parlodion (Mallenckrodt Chemical Works), drained for nine minutes, immersed in tap water and washed for two hours by suction. This process consistently gives membranes which hold back the streptococcus toxin. The toxin thus obtained usually contains about 100 Lf units per c.c. This Lf unit is, of course, based on the value attributed to our laboratory standard of 1300 U.S.A. units per c.c., and one Lf dose of toxin is equivalent to $\frac{1}{13.00}$ c.c. of our standard serum. A flocculating sub-standard serum, whose in-vivo value has been accurately determined by the skin test, has been introduced for convenience; it is a serum which has been concentrated by a peptic digestion method (Pope, 1938) and in common with most sera treated in this way flocculates rapidly, even with unconcentrated toxin. Concentrated toxin is used for titrating all sera of value greater than 100 units per c.c. The method of titration is almost identical with that employed by Ramon for diphtheria toxin-antitoxin flocculation. One c.c. amounts of concentrated toxin are titrated against varying quantities of serum over a 20 per cent. range in Dreyer tubes one-third immersed in a water-bath at 50° C. A 10 per cent. range may be employed, but titrations at 5 per cent. differences are difficult owing to simultaneous flocculation occurring in several tubes. For all the direct titrations a 20 per cent. range has been used, and the results obtained by interpolation may be regarded as accurate to about 10 per cent. The first tubes to flocculate give the value of the serum in terms of the toxin used.

Titration with unconcentrated toxin

Low value sera, below 100 units, are titrated by blending with a rapidly flocculating antitoxin by a method similar to that described by O'Meara (1935). A mixture of two parts of a rapidly flocculating serum of known value and one part of the serum to be tested are titrated against 1 c.c. of an unconcentrated toxin (containing about 20 Lf units per c.c.) first over a 20 per cent. and finally over a 10 per cent. range. Knowing the point of optimal flocculation of the mixture, the value of the tested serum can easily be calculated. Blending methods of titration are not accurate, and unless care is taken in every case to select for blending an antitoxin of approximately the same value as the test serum the results may be very

inaccurate. Consequently all flocculation results quoted in this paper for sera of below 100 units may be regarded as only approximate and of little value for comparative purposes.

EXPERIMENTAL OBSERVATIONS

Nature of the flocculation

The general physical characteristics of the streptococcus toxin antitoxin flocculation have been adequately described by O'Meara (1935) and Rane and Wyman and their observations have been confirmed in all major respects. It is of importance to stress the effect of dilution of toxin on flocculation. This is shown in table I and it is an obvious deduction that unconcentrated toxins prepared by the usual methods would be quite unsuitable for flocculation tests since, at the best they would only give a precipitate just visible to the naked eye after many hours at 50° C. This is the probable explanation of the difficulty experienced by early workers in obtaining flocculation in toxin antitoxin mixtures with anything approaching consistency.

TABLE I

The effect of toxin dilution on flocculation

Test dose of toxin (Lf value)	Optimal flocculation time (kf value)	Size and nature of flocculation
100.0	30 minutes	Numerous large floccules
50.0	70	
25.0	2 hours	Few large floccules
12.5	4	small
6.2	1 day	Few very small floccules just visible to the naked eye
3.1	2 days	Few very small floccules hardly visible to the naked eye but visible in an agglutinoscope
1.6	No flocculation	No flocculation

Nature of free toxin liberated by the destruction of antitoxin in toxin antitoxin floccules

One of the most conclusive pieces of evidence that the erythrogenic and flocculating antigens are identical is derived from the splitting of toxin antitoxin floccules. It has been found possible by the following method to destroy the antitoxin in streptococcus toxin antitoxin floccules and to liberate free toxin. The washed floccules are suspended in saline and heated in a boiling water bath for half an hour, they are then cooled and the floccules digested with trypsin at 50° C for three hours at pH 8.0. The mixture is heated to 100° C for a few minutes to destroy the trypsin and then filtered. The filtrate contains free toxin, but in view of the rather drastic treatment required, only a small yield of toxin amounting to 5-10 per cent is obtained. The method depends

on the relative stability of streptococcus toxin to heat and to the action of trypsin. The toxin derived from the splitting of the floccules has both flocculating and erythrogenic properties, and the unitage of the toxin as determined by flocculation and skin tests is the same. For example, in one experiment a toxin was obtained of which one c.c. had an Lf of 11.3 units and neutralised by the skin test a quantity of antitoxin equivalent to 13 units. These experiments seem to prove conclusively that the flocculating and erythrogenic toxins are identical.

Comparison of the erythrogenic and flocculating antibodies in fractionated serum

A scarlet fever antitoxin was fractionated by ammonium sulphate precipitation at different levels and the erythrogenic and flocculating values determined. It can be seen from table II that there is fair agreement between both values in all fractions and that the flocculating antibody is mainly concentrated in the pseudoglobulin fraction, as would be expected if it was similar to most antitoxins.

TABLE II

Skin test and flocculation values of fractionated serum

Fraction	Amount of (NH ₄) ₂ SO ₄ (g. per l.)	Protein content of final dialysed material (per cent)	Unitage by skin test	Unitage by flocculation test	In-vivo/in-vitro ratio
A (Plasma)	2000	1700	1.18
B (Serum)	1400	1700	0.82
C	0.210	15.36	700	1360	0.51
D	210-250	16.90	2100	4420	0.48
F	250-290	15.40	8000	8840	0.91
H	290-330	11.48	3300	4420	0.75
K	330-360	6.07	500	490	1.02
L	360-410	5.72	25	20	1.25

Comparison of the erythrogenic and flocculating antibodies of bleedings from horses taken at different stages of immunisation

The results of 240 bleedings from 78 horses at different stages of immunisation are summarised in table III. They represent a portion taken at random from a very large number which have been tested in these laboratories during the past eighteen months. The results on the whole indicate some correlation between the flocculation and skin test values. There is a small proportion of sera in which the difference appears to be greater than would be expected both from the experimental error of the titrations and the normal differences between in-vivo and in-vitro values of sera of varying degrees of avidity. Moreover it might be argued from the increase of the in-vivo/in-vitro ratio from 0.6 to 1.6 with increase of unitage

of sera from 100 to 500 units that this indicates two antibodies and that, as the course of immunisation proceeds, the erythrogeinic antibody increases at a greater rate than the flocculating antibody. The agreement also is not as close as that obtained by Rane and Wyman, using American-produced sera. There appear, however, to be reasonable explanations for the discrepancies and they will be referred to later. It is sufficient at the moment to indicate the type of agreement obtained and to observe that it is on the whole quite good.

TABLE III

Results of 240 bleedings from 78 different horses

In vivo value of bleedings in units	In vivo in vitro ratio	No. of bleedings in each group	Highest and lowest ratio in each group	Arithmetic average of ratio	Average
500 or more	>2 0 1.2 0.5 1 0	10 37 13	Highest = 4 0 Lowest = 0.5	1.6	
100-500	>2 0 1.2 0.5 1 0 <0.5	4 76 53 3	Highest = 3 0 Lowest = 0.3	1.2	1.2
100 or less	1.2 0.5 1 0 0.25 0.5 0.1 0.25	4 19 12 7	Highest = 1.4 Lowest = 0.11	0.6	

Comparison of the skin test and flocculation values of sera from individual horses during the course of immunisation

Horses are immunised with toxin produced according to the method described by O'Meara (1934). It is the custom in these laboratories to give the horses a preliminary course of injections with alum-precipitated toxin; this is followed, after a rest, by the main course of injections with toxin. When the horses have reached a satisfactory titre a number of bleedings of several litres are taken from them. The horses are rested for a few weeks and then given a rapid course of injections with alum precipitated toxin, bled again and rested. This latter course of injections is repeated and the horses are eventually killed by being bled for a maximum yield of blood, under general anaesthesia. These courses of injections are referred to as the preliminary, 1st, 2nd and 3rd courses respectively. For a variety of reasons horses may be killed before all the courses have been given.

Table IV shows the results from a number of horses illustrating the different kinds of response obtained. Before discussing these results it is necessary to consider the accuracy of the tests involved. The flocculation results may be regarded as accurate to 10 per cent. and the skin values to 50 per cent. Consequently variations of in-vivo/in-vitro ratios from about 0.6 to 1.7 are without significance, provided there is a normal scatter about the mean value. There

TABLE IV—*Showing the skin and flocculation values of sera from individual horses at different stages of immunisation*

Immunisation course	Date of bleeding	Skin value	Flocculation value	In-vivo/in-vitro ratio	Immunisation course	Date of bleeding	Skin value	Flocculation value	In-vivo/in-vitro ratio
Horse 9017					Horse 8743				
1	29.7.40	2.0	1.9	1.06	1	17.1.40	0.1	0.3	0.37
	5.8.40	2.0	2.3	0.89		1.2.40	0.4	0.3	1.21
	12.8.40	4.0	1.9	2.12		8.2.40	0.75	0.5	1.50
	19.8.40	4.0	3.2	1.23		9.2.40	0.5	0.5	1.06
	26.8.40	4.0	3.0	1.34		9.3.40	2.0	2.1	0.96
	2.9.40	6.0	3.2	1.85		18.3.40	1.5	1.6	0.96
	9.9.40	6.0	3.9	1.51		29.3.40	2.0	1.3	1.54
2	16.9.40	8.0	5.6	1.41		15.4.40	2.0	1.6	1.27
	14.10.40	10.0	6.8	1.48		22.4.40	2.0	2.3	0.89
3	14.11.40	12.0	3.9	3.08	2	2.5.40	2.0	2.3	0.89
						3.6.40	8.0	3.8	2.12
						1.7.40	8.0	3.1	2.58
Horse 9020					Horse 8742				
1	15.7.40	0.5	1.3	0.38	1	7.2.40	0.4	3.2	0.13
	5.8.40	0.4	1.3	0.31		10.2.4	1.5	3.2	0.46
	12.8.40	1.0	2.3	0.44		21.2.40	2.5	3.2	0.77
	19.8.40	2.0	3.2	0.62		24.2.40	3.5	3.2	1.09
	26.8.40	3.3	3.9	0.85		13.3.40	6.0	3.2	1.87
	2.9.40	4.5	3.9	1.15		16.3.40	6.0	3.2	1.87
	9.9.40	8.0	3.9	2.36		20.3.40	13.0	4.6	2.18
2	16.9.40	7.0	3.2	2.16	2	27.3.40	6.0	4.5	1.33
3	21.10.40	6.0	2.3	2.66		30.4.40	16.0	14.3	1.12
	25.11.40	3.0	4.7	0.64	3	4.6.40	10.0	8.0	1.25
Horse 8997					Horse 8989				
1	8.7.40	0.4	0.9	0.46	1	8.7.40	0.4	0.8	0.51
	22.7.40	1.5	1.3	1.14		5.8.40	1.0	1.6	0.62
	31.7.40	2.5	1.6	1.39		19.8.40	3.0	2.11	1.45
	14.8.40	8.0	2.5	3.22		2.9.40	10.0	2.7	3.5
2	21.8.40	10.0	3.0	3.35	2	30.9.40	12.0	9.3	1.3
	18.9.40	12.0	6.2	1.94					
Horse 9239					Horse 8986				
1	7.10.40	1.0	1.8	0.57	1	8.7.40	0.3	0.9	0.32
	21.10.40	4.0	3.9	1.03		5.8.40	1.2	1.6	0.74
	4.11.40	8.0	6.0	1.33		19.8.40	4.0	2.7	1.48
2	2.12.40	12.0	13.5	0.89		2.9.40	6.0	3.9	1.54
	6.1.40	16.0	13.5	1.19		30.9.40	7.0	6.8	1.04
3						4.11.40	5.0	3.9	1.28
						9.12.40	7.0	5.2	1.35
Horse 8904					Horse 9240				
1	26.6.40	0.3	2.7	0.11	1	7.10.40	3.0	2.3	1.3
	24.7.40	4.0	5.6	0.71		21.10.40	5.0	3.6	1.4
	3.8.40	10.0	6.5	1.55		4.11.40	8.0	5.11	1.55
2	3.9.40	10.0	11.2	0.89	2	9.12.40	14.0	11.2	1.24

Values are given as units /100.

are, however, a number of discrepancies between the skin and flocculation values which are apparent from the examples shown in table IV. Firstly there is often a very low *in vivo/in vitro* ratio at the beginning of the main (1st) course of immunisation (see horses 8904, 8986, 9020). This happens with about one half of the horses. In a few cases (horse 8742) the flocculation titre remains constant for a period during which the skin value shows a steady rise, this is not due to a completely non specific flocculation, since normal horse sera do not flocculate with the antigen. Eventually as immunisation proceeds the skin and flocculation values agree more closely. Secondly, there is an impression gained from some horses (8742, 8743, 8989, 8997) that the skin value may rise fairly rapidly and the flocculation value remain low and later rise to the same value as the skin test. This may or may not be associated with a change in the course of immunisation. This variation of the *in vivo/in vitro* ratio with different bleedings from the same horse is very difficult to explain. Inaccuracy of testing and questions of avidity do not appear to offer satisfactory explanations. There remains the possibility of difference in quality of antitoxin which affects the *in vivo/in vitro* ratio. This is pure speculation but it is of interest that treatment with pepsin destroys the high ratios. For example, with one serum before treatment with pepsin the ratio was 3.2, and afterwards 0.97. Moreover it can be seen from table V that sera after treatment with pepsin show a much closer agreement between skin and flocculation values than the same sera before treatment. The possible reasons for this discrepancy are a subject for further investigation, the main purpose is to recognise these differences and make allowance for them in interpreting the flocculation results. Approximately one half of the horses are similar to horses 9240, 9239 and 8986 in which the skin and flocculation values agree during the whole period of immunisation.

Flocculation values of concentrated sera

Concentrated sera have been tested for flocculation values, they consist of mixtures of bleedings from different horses which have been concentrated by a peptic digest method to give sera of values from 2000 to 7000 units per c.c. The agreement between the skin and flocculation values is closer than that obtained with unconcentrated sera. This is to be expected, since individual variations tend to be counterbalanced during blending and peptic digestion eliminates the high *in vivo/in vitro* values found in the sera of some horses. In a group of twenty two consecutive batches of concentrated sera the average *in vivo/in vitro* ratio was 1.1 and the limits of variation were from 0.73 to 1.9. These variations are almost exactly what would be expected from the limits of accuracy of the skin test.

*Yields of antitoxin after concentration by peptic digestion
calculated on skin test and flocculation values*

The flocculation value of scarlet fever antitoxin can be determined much more accurately than the skin value, since the former is determined on a 10 per cent. and the latter on a 100 per cent. range. It is of interest to calculate the yield of antitoxin obtained after concentration, based on the skin and flocculation values. These sera are concentrated as a routine by the peptic digestion method described by Pope (1938): the results are given in table V.

TABLE V
Antitoxin content of sera before and after concentration

Batch	Mixed plasma before pepsin		pepsin treatment concentration		Percentage yield calculated from skin test	Percentage yield calculated from flocculation test
	Skin value	Flocculation value	Skin value	Flocculation value		
54	380	210	4300	4840	32.8	74.7
55	100	80	2500	2120	65.1	69.1
56	350	260	5000	5830	54.1	70.8
58	400	260	6000	5860	42.6	64.1
60	350	320	6000	4950	54.3	49.1
62	500	390	6500	8580	31.4	53.3
64	100	90	1600	2200	45.5	67.2
65	300	160	4500	5400	40.3	87.3
66	100	70	2000	1280	58.8	54.0
67	400	350	9000	6840	81.5	70.8
70	110	130	3100	2750	91.6	65.6
71	130	60	2000	1480	47.4	74.1
73	400	233	5500	5180	45.9	72.0
74	80	60	1800	1450	59.0	62.0
75	280	210	5200	5100	53.0	69.2
77	400	190	6600	5240	36.3	60.8
80	300	230	4500	4280	49.8	63.0
81	100	70	1700	1690	52.8	71.7
82	270	160	3000	3140	42.2	75.9
84	300	160	4000	3890	46.6	84.8
85	170	110	2700	2810	43.3	67.7

Values in U.S.A. units.

They provide confirmatory evidence that the skin and flocculating antibodies are probably the same, since they are obviously concentrated by the process in similar ways. There are two further points of great importance. Firstly, the yields calculated on the flocculation values are in keeping with the results obtained in these laboratories with other types of sera, both as regards yield and the relation of the yield to the protein figures before and after digestion. The yields calculated on skin values show a much bigger variation than would be expected and in some cases they appear highly improbable. It would seem that the flocculation values are a closer approximation to the truth. Secondly, the differences

between skin and flocculation values are almost entirely confined to the mixed plasma before treatment with pepsin and concentration. Apparently the skin test is less accurate, giving higher values to those sera unmodified by pepsin and having a higher protein content. It is possible that pepsin either destroys some substance interfering with the skin test or modifies the antitoxin in some way which alters the in-vivo/in-vitro ratio.

Sera from different sources

A number of sera from various sources unconnected with these laboratories have been tested by the skin and flocculation methods (table VI). No attempt has been made to correlate the values with the unitage stated on the labels, as some of the sera are many years old. It is of interest to note that there is reasonable agreement between the in-vivo and in-vitro values in every case. That sera produced over a long period in different countries should give such agreement is strong evidence that the two antibodies are identical.

TABLE VI

Skin and flocculation values of sera from different sources

Source	Skin value	Flocculation value	In vivo In vitro ratio
American	40	35	1 17
"	350	460	0 76
"	500	350	1 42
"	600	600	1 0
"	800	520	1 54
"	1600	870	1 84
"	2500	2080	1 20
"	3300	1800	1 83
British	400	260	1 54
"	600	460	1 30
Japanese	650	460	1 44
Hungarian	300	260	1 15

Ratio of the quantities of toxin flocculating with and neutralised by one unit of antitoxin

With the skin test there is always included on each rabbit a series of mixtures of toxin and standard serum diluted to contain 100 U.S.A. units per c.c. for direct comparison with the sera to be tested. These mixtures consist of 0.5 c.c. of toxin containing approximately 2 million skin test doses per c.c. mixed with varying quantities of the standard scarlet fever antitoxin dilution. The end-point in most rabbits corresponds to 0.4 c.c. of antitoxin, and consequently 1 unit of antitoxin is equivalent to 25,000 S.T.D. or a volume of 0.0125 c.c. With the flocculation test, 0.4 c.c. of scarlet fever antitoxin containing 48 units per c.c. is equivalent to 1 c.c.

of toxin containing 2.5 million S.T.D. per c.c., or 1 unit of antitoxin is equivalent to 125,000 S.T.D., that is, to a volume of 0.0625 c.c. of a toxin containing 2 million S.T.D. per c.c. The ratio of the volume of toxin flocculating with 1 unit of antitoxin to the volume neutralised by 1 unit of antitoxin in the skin test is 5 : 1. This is easily illustrated by injection into the rabbit of a range of dilutions covering the flocculation and skin neutralisation points, when it can be seen that it requires per unit volume of toxin five times as much serum to neutralise the skin reaction as it does to give optimal flocculation. This ratio of 5 : 1 is very different from the ratio of 1000 : 1 given by Rane and Wyman. No explanation of this difference is at the moment available ; however, it is almost entirely in the animal test. This ratio of 1000 : 1 is based on 1 unit of antitoxin being equivalent to 50 S.T.D., whereas with the skin test in these laboratories it is found to be equivalent to 25,000 S.T.D. The value of 125,000 S.T.D. found to flocculate with 1 unit of antitoxin is in close agreement with the 60,000 S.T.D. given by Rane and Wyman, since the skin test dose is a unit difficult of comparison between two laboratories. The immunological significance of this 5 : 1 ratio may be that we are dealing with antitoxins of extreme non-avidity, and that a very large excess of antitoxin is necessary to overcome the effect of dissociation. This 5 : 1 ratio is higher than those usually experienced in immunology, but the ratio of 1000 : 1 based on 1 unit of antitoxin being equivalent to 50 S.T.D. is so completely divorced from anything previously observed in serology as to render any explanation based on avidity highly improbable.

DISCUSSION

The main purpose of this investigation has been the purely practical one of determining whether the skin test can be replaced by a flocculation test. It is a natural corollary that this would only be satisfactory if the two antigens were identical. I think it can be considered proved that there is a specific flocculation associated with the neutralisation of the erythrogenic antibody. It has been seen that, although there are differences between the skin and flocculation titres of sera, there is nothing that rules out this possibility. Much of the evidence for the identity of antigens is inferential and it is seldom possible to give direct proof. Evidence such as that found by the destruction of toxin in diphtheria toxin-antitoxin floccules, leaving purified antitoxin which has both flocculating and animal-protecting properties, is striking confirmation of the current unitarian conception of serum reactions. The liberation of toxin from scarlet fever toxin-antitoxin floccules is conclusive evidence that there is a specific flocculation associated with the neutralisation of the erythrogenic toxin by antitoxin. In

general, with avid sera, the animal test is regarded as more accurate than a flocculation test, largely because more is known of the factors influencing the flocculating reaction. Like all flocculation tests, scarlet fever flocculation is subject to the well known errors due to non-specific flocculation, zone formation, one-sided spread of the zone of flocculation (indicating two optimal points of flocculation too close together to give zones) and the existence of sera which take a very long time to flocculate. On the whole this test seems fairly free from these difficulties. There is usually a zone of non-specific flocculation but normally it only appears after one to two days' incubation. It is far removed from the specific zone and the floccules are much smaller. It is probably due to bacterial protein, since the antigen is present in approximately the same amount in filtrates from all the strains of pathogenic streptococci examined, including those producing very little erythrogenic toxin. Very occasionally two zones of flocculation occur close together and more often there is a one-sided spread of the zone of flocculation. With the skin test there are also a considerable number of difficulties and an examination of the literature will readily reveal the trouble experienced in titrating scarlet fever antitoxins in view of the lack of a really susceptible experimental animal. The agreement between the in-vivo and in-vitro values has therefore to be considered in the light of a somewhat unsatisfactory animal test and a flocculation test which has the disadvantages usually associated with such methods. Under these circumstances the agreement between skin and flocculation values appears to be good and in fact evidence such as that obtained by the calculation of yields on concentration indicates that the flocculation test may be a closer approximation to the truth. Remembering the types of response obtained with different horses as shown in table V, the flocculation method may with advantage replace the skin test for many of these determinations. One observation, for which at the moment there is no precise explanation, is the variation of the in-vivo/in-vitro ratio with different bleedings from the same horse. This variation is apparently eliminated by treatment with pepsin.

SUMMARY

1. Haemolytic streptococcus (scarlet fever) toxin can be recovered from toxin-antitoxin floccules by the action of heat and trypsin. The recovered toxin has both erythrogenic and flocculating properties.

2. It is confirmed that there is specific flocculation associated with the neutralisation of toxin by antitoxin and that the reaction may be used for titrating the antitoxin.

3. Some horses may at one period during the course of immunisa-

tion show a high in-vivo/in-vitro ratio. This ratio is altered by treatment with pepsin, the in-vivo value coming much closer to the in-vitro value.

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THE BACTERIOLOGICAL EXAMINATION OF WATER SAMPLES WITH REFERENCE TO DIRECT AND SECONDARY INCUBATION AT 44° C.

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IS an effort to overcome certain objections to the technique officially recommended (Ministry of Health, 1934) for estimating *coli aerogenes* bacteria in water, Wilson and his colleagues (1935) described four methods for the quantitative examination of milk, water or other liquid material. Method II is purely a plating method and need not be considered further. The other three methods involve preliminary enrichment in MacConkey's bile salt lactose broth. Generally one 50 ml and five 10 ml quantities of fluid are inoculated into equal amounts of double strength MacConkey's broth and five 1 ml and five 0.1 ml quantities into 10 ml of MacConkey's broth of ordinary strength, but in special cases smaller amounts may also be used.

In *method I* the inoculated tubes are incubated for 48 hours at 37° C and a loopful of the culture of the highest dilution showing acid and gas is spread on a MacConkey's agar plate. This is incubated for a further 24 hours and three colonies are picked off for differentiation of the *coli*, intermediate and *aerogenes* types. The total number of living coliform organisms is estimated, preferably with the help of probability tables, and the proportion of the various types is determined from the results of the colony examinations. In *method III* one set of tubes is incubated at 37° and a second at 44° C for 48 hours. All positive tubes at 37° C are subcultured into citrate medium, which is examined for growth after 48 hours at 37° C. The probable number of coliform organisms is estimated from the MacConkey's tubes positive at 37° C. Those which yield growth on subculture in citrate are considered to contain organisms of the intermediate, *aerogenes* or *cloacae* type (I A C), while those which do not are regarded as containing only *Bact coli* I or II. The probable number of *Bact coli* I is estimated from the MacConkey's tubes positive at 44° C. In *method IV* one set only of MacConkey's tubes is incubated at 37° C for 48 hours. All positive tubes are subcultured into both citrate at 37° C and MacConkey's broth at 44° C and incubated for 48 hours. The probable number of coliform organisms is obtained from the original MacConkey's tubes positive at 37° C, the *Bact coli* I count from the number of confirmatory MacConkey's tubes showing acid and gas at 44° C, and the I A C count from the positive citrate tubes at 37° C.

Method I has been described by Wilson *et al* as tedious and presenting an erroneous picture and Bardsley (1938) came to the same conclusion. Method III is described by Wilson *et al* (p. 208) as being "peculiarly suitable for a rapid estimation of the faecal *coli* count. It is less satisfactory for determining the *coli aerogenes* ratio", while method IV is recommended by them if the presumptive coliform count, the faecal *coli* count and the I A C count are desired, and is of particular value in determining the *coli aerogenes* ratio.

The Ministry of Health (1939) express the opinion that organisms of the I.A.C. group are of no practical interest in the presence of more than minimal numbers of *Bact. coli*, and that it is only when *Bact. coli* is absent that the presence of other types of coliform bacteria becomes important. It should be noted, however, that Taylor (1941) found greater relative proportions of I.A.C. to faecal *coli* in polluted than in relatively pure waters. If the Ministry's view is correct, the *coli-aerogenes* ratio has little practical value, and the fact that method III is not satisfactory for the determination of this ratio, becomes of little account. As method III seems suitable for the rapid determination of faecal *coli* and method IV the most satisfactory for I.A.C., a combination of these methods should provide a still more reliable test. The work involved would be little more than that required for method I, already long established in this country. Moreover the relative accuracy of method IV is left intact, and the speed with which the *Bact. coli* count can be obtained may often be usefully applied in the issuing of interim reports, the value of which will be particularly appreciated at the present day.

Opportunity has been taken to apply the combined test to a considerable number of routine samples of waters of different types. The data thus obtained throw light on the relative merits of primary incubation at 44° C. as opposed to the incubation at this temperature of secondary tubes, that is, tubes inoculated from primary cultures at 37° C.; for there is a possibility that *Bact. coli* may suffer from "heat-shock" and that on this account the primary cultures at 44° C. may not give as high a count as that yielded by secondary cultures at this temperature. Raven *et al.* (1940) compared the presumptive *Bact. coli* counts at 37° and 44° C. on 329 samples of water, but in only 135 samples were *Bact. coli* present in both tests. Their results showed a somewhat higher number of *Bact. coli* developing at 37° than at 44° C. but in the majority of samples the differences may well have been due to chance. They declined to draw any conclusions, but suggested that further study of the presumptive test at 44° C. seemed desirable.

TECHNIQUE

The usual quantities of water were inoculated into two sets of MacConkey's broth tubes (2 per cent. peptone, 0.5 per cent. sodium tauroglycocholate, 1 per cent. lactose, 1 per cent. Andrade's indicator), one set being incubated at 37° C., the other in an accurately controlled water-bath at 44° C. (Clegg and Sherwood, 1939). The tubes incubated at 44° C. were not pre-heated before inoculation as this had been found unnecessary (Clegg and Sherwood). Both sets were examined after 24 and 48 hours and subcultures were made from all tubes showing acid and gas.

The positives at 37° C. were subcultured in Koser's citrate medium containing 0.008 per cent. brom-thymol-blue at 37° C., and in MacConkey's broth at 44° C. A loopful was also spread on a segment of an eosin-methylene blue (E.M.B.) agar plate. The inoculation of MacConkey's broth at 44° C. was performed with a triple loop—three loops on one wire—as there seems to be some doubt whether minimal inocula suitable for use at 37° C. will suffice for tubes incubated at 44° C. (Raven *et al.*).

Positive primary cultures at 44° C were subcultured in citrate medium and on EMB plates in order to detect organisms other than *Bact coli* which might have fermented MacConkey's broth at 44° C

There are three quite distinct colonial types of *Bact coli* on EMB plates, each exhibiting certain variations, which the water bacteriologist may encounter and should be able to identify. Type I, most commonly observed, is 2.3 mm in diameter, flat and dry, with a large dark centre and showing little tendency to confluence, it may or may not exhibit a metallic sheen or a target marking in the centre. Type II is 4.5 mm in diameter, flat and dry, with a roughened surface and irregular edge, it has a relatively smaller darkened centre than type I and may or may not exhibit a metallic sheen. Type III is about 3 mm in diameter and is the least common, it is mucoid, raised and smooth, with a dark centre, and exhibits a distinct tendency to confluence, it may or may not exhibit a metallic sheen. Levine (1921) claims that colonies of type II constitute only 2 or 3 per cent of the *Bact coli* colonies observed. The experience of the author is that in this country they are more numerous.

RESULTS

In all, 837 samples of water of different types from the North Wales area were examined. The distribution of types is shown in table I.

Of those samples containing coliform organisms (556) 44.2 or 79.5 per cent contained faecal *coli*. This figure is just outside the range of 80-90 per cent found by Bardsley (1934) and subsequently quoted by the Ministry of Health (1939) for waters in this country. But the findings in any given series would depend on the types of water examined and if a high proportion of these were chlorinated or well waters the percentage figure might be well below 80-90.

Results of confirmatory tests on primary tubes positive at 37° C

In all the samples in table I, 4065 tubes showed acid and sufficient gas to fill the concavity of the Durham's tube when incubated at 37° C. Of these, 2573 (63.3 per cent) were positive at 24 hours, and 1967 (76.4 per cent) of the latter were proved to contain either *Bact coli* type I or irregular type II. Of the remaining 1492 (36.3 per cent of the total) positive at 48 hours, only 255 (17.1 per cent) contained these organisms. Raven *et al* found a somewhat higher proportion of their positives at 24 hours to contain *Bact coli*, but their samples were derived largely from filtered and some from chlorinated waters, whereas the sources of supply for this work were extremely varied and there were many samples of well water which gave high coliform counts and very few or no faecal *coli*.

Anomalous reactions at 37° C were largely caused by cultures giving colonies typical of *Bact coli* on EMB plates but negative reactions in citrate and at 44° C. Only 92.49 per cent of the *coli* like colonies on the EMB plates were accompanied by positive

TABLE I

Results of the examination of 837 samples of water from North Wales

Type of water	No. of samples examined	No coliform bacteria found		Coliform bacteria found		Coliform bacteria but no <i>Bact. coli</i> found		<i>Bact. coli</i> found		No. incompletely examined *	No. available for statistical analysis (see p. 60)
		No.	Percent.	No.	Percent.	No.	Percent.	No.	Percent.		
Chlorinated water (different types) . . .	243	161	66.3	82	33.7	30	12.4	52	21.3	1	50
Untreated piped supplies . . .	185	55	29.7	130	70.3	30	16.2	100	54.1	6	96
Untreated streams, springs, rivers and surface waters	150	16	10.7	134	89.3	12	8.0	122	81.3	5	114
Filtered water . . .	120	24	20.0	96	80.0	9	7.5	87	72.5	4	82
Untreated well water . . .	96	18	18.8	78	81.2	29	30.2	49	51.0	9	43
Untreated lake and reservoir water . . .	20	4	20.0	16	80.0	1	5.0	15	75.0	2	13
Untreated sea water . . .	23	3	13.0	20	87.0	3	13.0	17	74.0	2	15
Total . . .	837	281	33.6	556	66.4	114	13.6	442	52.8	29	413

* Primary incubation at 44° C. was omitted through lack of incubator space.

reactions at 44° C. in the confirmatory tube. One hundred and seven cultures which did not give this confirmatory test were examined in detail (table II).

TABLE II

Reactions of 107 anomalous strains from primary cultures at 37° C.

Results of tests on pure cultures														
No gas at 44° C					Limited gas* at 44° C					Good production of gas at 44° C.				
No	I	M R	V P	C	No	I	M R	V P	C	No	I	M R	V P	C
20	+	+	—	—	5	+	+	—	—	46	+	+	—	—
19	—	+	—	+						2	—	+	—	—
8	—	+	—	—										
3	—	—	+	+										
2	+	—	—	—										
2	—	+	+	+										

* Insufficient to fill the cavity of the Durham's tube

I. = indole, M R = methyl red, V P = Voges-Proskauer, C = citrate

There is nothing very surprising in the first two categories in table II. Strains of *Bact. coli* which give limited or inconstant gas formation in MacConkey's broth at 44° C. are observed on rare occasions. The 20 cultures which gave the reactions, indole+, M.R.+, V.P.—, citrate—, are of the type called by Wilson *et al.* "Irregular *coli*-like I", and by the Ministry of Health (1939) "Irregular type I".

The cultures in the third category failed to produce acid and gas at 44° C. in the ordinary confirmatory test, but when the organisms were isolated in pure culture they did so, and they continued to do so in repeated tests. These results were not due to inaccurately controlled water-baths or to the use of hot wires in making the initial subcultures. They might conceivably have been due either to the need for time in which the enzymes could become adapted to the high temperature or to the presence of an inhibitory organism in the culture from the primary growth, but of this there is no evidence.

There is, however, some evidence that tubes incubated at 44° C. may require larger inocula than tubes incubated at 37° C. Raven *et al.* drew attention to this fact in connection with experiments on *Bact. coli*. For this reason, as mentioned above, we have used a triple loop in making inoculations into tubes for incubation at 44° C. Even so, it is quite possible that, on occasion, insufficient numbers of organisms were transferred. Although Wilson *et al.* found no significant difference in the number of *Bact. coli* which developed when parallel tubes of MacConkey's broth were incubated at 37° and 44° C., this has not been the experience of the author on the

few occasions when this has been tested. Table III gives evidence that a temperature of 44° C. is frequently not as suitable as 37° C. for the growth of *Bact. coli*.

TABLE III
Growth of Bact. coli in MacConkey's broth at 37° and 44° C.

Strain	Viable counts (millions per ml.) on MacConkey's agar			
	Broth incubated 24 hours		Broth incubated 48 hours	
	37° C.	44° C.	37° C.	44° C.
A	680	240
B	810	260
C	480	23	100	1.6
D	500	5	147	2.7
E	490	7
F	380	26
G	470	1.2	176	0.36
H	590	1.2	172	1.6

Certain other cultures produced colonies typical of *Bact. coli*, apparently in pure culture, on the E.M.B. plate and gave positive reactions both at 44° C. and in citrate in the confirmatory test. When the colonies were picked off, all but one proved to be citrate—. In other words the organisms responsible for the growth in citrate in the confirmatory test were not visible on the E.M.B. plate, but their presence was confirmed in the citrate tube. As only one colony from each culture was tested, it is quite possible that other colonies existed, giving the same appearance as those tested, which were both citrate+ and 44° C.+. In view of the number of tests made (38) this is thought improbable. The one culture which was citrate+, 44° C.+, and gave colonies typical of *Bact. coli* was a strain of *Bact. aerogenes* I, derived from a primary tube which was positive at 48 hours.

In this country the incidence in water of organisms giving a positive result both in MacConkey broth at 44° C. and in citrate medium appears generally to be low. In India, Raghavachari and Iyer (1938-39) reported that 60-70 per cent. of organisms similar to *Bact. aerogenes* fermented MacConkey's broth at 44° C. and concluded that incubation at 44° C. as a test for faecal *coli* can only be regarded as valid if it can be proved that the organism called "Irregular IV" is a normal inhabitant of the Indian intestine. Recently Harding (1940) made a similarly adverse report based on the examination of water in the North-East Derbyshire area; this appears to be the first really serious complaint against incubation at 44° C. in this country. Raven *et al.* on occasion isolated *Bact. aerogenes* which fermented MacConkey's broth at 44° C., but their experience was that such cases were peculiar to certain sources of supply. Small proportions of these organisms have also been found in polluted shellfish, but the distribution was more general (Clegg and Sherwood). The general opinion in this country appears to be that incubation at 44° C. is a more specific test for *Bact. coli* than is growth in MacConkey's broth at 37° C. for coliform organisms.

Results of confirmatory tests on primary tubes positive at 44° C

There were 1766 primary tubes showing acid and gas at 44° C. Of these, 42 (2.4 per cent) failed to show colonies typical of *Bact coli* on the EMB plate, 30 gave no growth on the EMB plate and 12 showed atypical colonies, 10 of which, when examined in pure culture, gave the reactions shown in table IV. Six other primary tubes which showed an alkaline reaction and gas yielded no growth on the EMB plate.

TABLE IV

Reactions of pure cultures of 10 anomalous strains from primary cultures at 44° C

No	Indole	M I	V P	Citrate	Gas at 44° C	Remarks
3	—	+	—	—	—	Coccus, small dark colonies ca 1 mm
3	+	+	—	—	—	
1	—	—	+	—	—	Small watery colonies
1	+	—	—	—	—	
1	+	+	+	—	(Alk) +	
1	+	+	—	—	+	

Four of the anomalous cultures gave the reactions of *Bact coli* although only one of them produced acid and gas at 44° C. Absence of growth on EMB plates gave rise to the majority of these anomalies. It has been noted in this work that certain cultures of *Bact coli* will not remain alive in MacConkey's broth at 44° C for 48 hours, but it is not possible to say whether the failure to grow on EMB plates was due to the death of *Bact coli* or to the fact that anaerobes were responsible for the production of gas in the primary tubes.

Comparison of methods III and IV

For the comparison of the most probable numbers of *Bact coli* obtained by methods III and IV a fairly comprehensive idea of the situation can be obtained from the figure (p 58). This has been drawn on a logarithmic scale to keep it within reasonable compass. The diagonal line is where the points should fall if both methods yielded the same result and if the dilution method of examination did not admit of such wide variations (Ministry of Health, 1939). In drawing this diagram a difficulty was encountered in plotting pairs of numbers, one of which was 0. Clearly this cannot normally be done on a logarithmic scale, but the number of such samples (104) made it imperative that they should be included. A suggestion, put forward by Mr Buchanan Wollaston, of plotting values of 0 in a special space labelled "less than 1", was adopted. The diagram shows (a) that there is a fairly even distribution of the extent of pollution in the waters examined, (b) that the limits of variation are

approximately the same on both sides of the line, and (c) that the larger number of points falls on the "method IV" side of the line.

It is not possible to show in the diagram the extent of zoning of the various types of water, but as might be expected, the chlorinated, filtered and raw piped waters largely fill up the bottom half of the diagram while the raw water from streams, springs, rivers and surface waters predominate in the higher numbers, the other types being more or less dispersed about the middle. The diagram, moreover, shows the grave difficulties involved in the application of mathematical analysis.

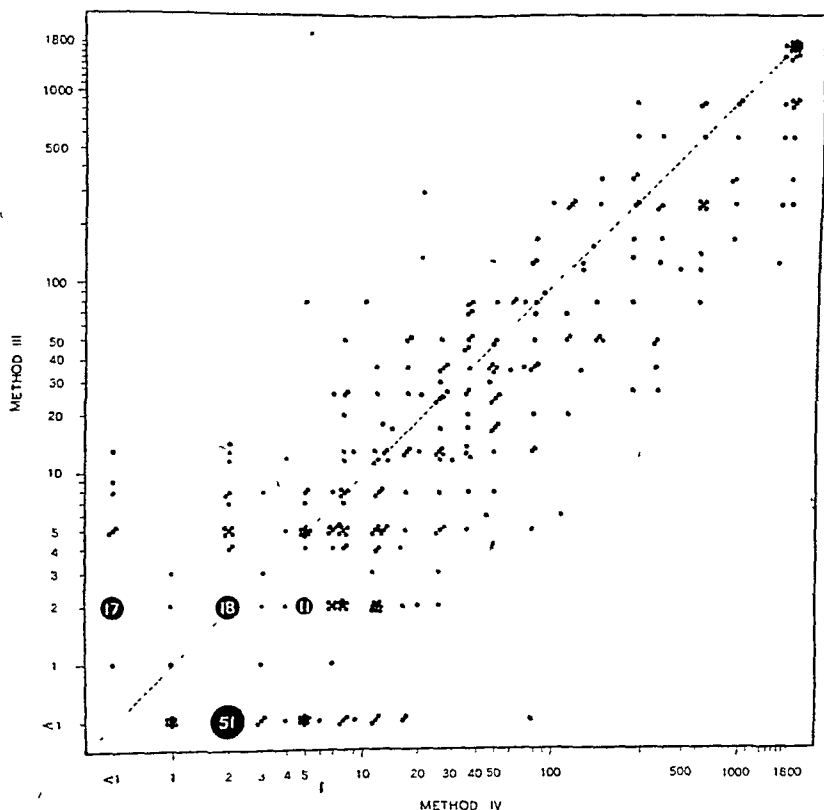


FIG.—Comparison of the results of examination of 413 water samples by methods III and IV expressed as probable numbers of *Bact. coli* per 100 ml.

It will be seen in table V that in none of the types of water do the figures obtained by method III exceed those given by method IV.

Although there is a great difference in the number of times each method exceeds the other ($\text{III} > \text{IV}$ 104 times; $\text{IV} > \text{III}$ 249 times; ratio 41.8:100), the difference in the average probable number of *Bact. coli* yielded by both methods is not great ($\text{III} : \text{IV} = 72.6 : 100$). Thus although IV is in excess of III on a great many occasions the amount of this excess is not large.

TABLE V

Comparison of results obtained in the examination of water by methods III and IV

Type of water	No of samples	III>IV		III=IV		III<IV		Total probable Bact coli		Average probable Bact coli per 100 ml		Probable Bact. coli by method III as a percentage of the no. by method IV
		No	Per cent	No	Per cent	No	Per cent	III	IV	III	IV	
Stream, spring, river and surface	114	30	26.3	20	17.5	64	50.2	35,192	43,750	308.7	383.8	80.4
Raw piped	96	27	28.1	17	17.7	52	54.2	2224	2497	23.2	26.0	89.1
Filtered	82	18	22.0	6	7.3	58	70.7	4315	8963	52.6	109.2	48.2
Chlorinated	50	11	22.0	5	10.0	34	68.0	485	763	9.7	15.3	63.6
Well	43	11	25.6	7	16.2	25	58.2	7895	11,770	183.6	273.7	67.1
Lake and reservoir	13	4	30.8	2	15.4	7	53.8	617	727	47.5	55.9	84.9
Sea	15	3	20.0	3	20.0	9	60.0	1423	3386	94.9	225.7	12.0
Total	413	104	25.2	60	14.5	249	60.3	52,151	71,856	126.3	171.0	72.6

As yet no satisfactory statistical test has been evolved to test the significance of any difference between two bacteriological tests performed by the dilution method. The reason for this is the very large experimental error of the dilution method (Ministry of Health, 1939). However, Mr Buchanan Wollaston, who is shortly to publish a statistical method for comparing paired results arrived at by the dilution method, has been good enough to study the results obtained by using methods III and IV in parallel and the gist of his observations is as follows (personal communication).

Of the 413 samples in which *Bact. coli* was shown to be present by one or both of the methods there are 353 pairs of counts in which these yielded different results. Of these, method IV is greater in 249 cases and method III in 104 cases. The exact probability of obtaining as great an excess of positive (or negative) signs, if the chances of a positive is equal to a half and equal to that of a negative, would be found by adding up all the terms of the binomial $(\frac{1}{2} + \frac{1}{2})^{353}$, outside and including the term corresponding to "249 heads, 104 tails" and outside and including the term "249 tails, 104 heads". This would be a very lengthy calculation and, as will be shown later, is not necessary. When n is as great as 353, the binomial is closely approximated to by the normal distribution with the same mean and standard deviation, especially as the binomial in question is symmetrical. The standard deviation is equal to $\sqrt{\frac{1}{4} \times 353}$ or about 9.4. The observed difference from the mean of the distribution, which is 176.5, is 72.5. The difference from the mean is therefore nearly eight times the standard deviation. Pearson's tables (1930) show that seven times the standard deviation on both sides together is only exceeded about once in 250,000,000,000 trials by chance. Thus without any further test this quite definitely proves that method IV gives a greater calculated most probable number than method III.

When the pairs of results are tested separately only 64 can possibly be regarded as being significantly different. Of these method IV is greater in 51 cases and method III greater in 13 cases. This confirms the general test of the signs.

The above results give no indication as to the extent of the difference. However, the main question, whether method IV gives a greater count than III, has been answered, and this is the first step towards solving the problem.

DISCUSSION

As method III gives an average figure consistently lower than method IV it may be argued that the less efficient method should be discarded. This is a view with which the author does not agree for the following reasons. In 25 per cent. of the samples method III gave a higher count than method IV. This percentage can hardly be ignored. Moreover, method III will almost invariably give a result overnight, whereas method IV requires 3 and sometimes 4 days. Speed in issuing a report is always an important consideration but in present-day conditions it is often of paramount importance.

Thus although method III suffers from the obvious disadvantage of giving a smaller count than method IV in 60.3 per cent. of the samples examined, this disadvantage is somewhat outweighed by the other considerations. The author's opinion is that method III is an invaluable addition to method IV and that the greater speed and accuracy obtained by running both tests in parallel counterbalance the extra labour involved.

The reason why method IV gives a greater count than method III in so many cases is not clear and an investigation into this problem might throw light on the way in which *Bact. coli* reacts to living conditions at 44° C. and help towards establishing a standard and more rational water test.

SUMMARY

1. The four methods of bacteriological examination of water described by Wilson *et al.* (1935) are summarised and their relative merits discussed. An attempt is made to show that a combination of the two best methods (III and IV) would yield a still more reliable test than the best method (IV) alone.

2. The results of examining 837 samples of water of all types from the North Wales area are discussed.

3. The results of the confirmatory tests on the primary cultures at 37° and 44° C. are discussed and certain anomalous results are considered in detail.

4. A comparison between direct and secondary incubation at 44° C. (methods III and IV) is made. Of the 837 samples, 413 showed *Bact. coli* by one or both methods. The ratio of total probable *Bact. coli* yielded by method III to that obtained by method IV is 72.6 : 100. In 353 out of 413 samples the probable number of *Bact. coli* obtained by the two methods differed. Statistical analysis shows that, although the proportion of samples in which the results differ significantly is small, the number in which method IV gave a higher count than method III is significant.

5. It is suggested that, while method IV gives, on an average, a higher probable count than method III, a combination of both methods should still be used in view of the speed with which results can be obtained by method III.

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DIPHTHERIA PROPHYLAXIS: MODERN METHODS OF PREPARING AND ASSAYING PROPHYLACTIC AGENTS, WITH A NOTE ON THE ESSENTIAL NATURE OF TOXOID-ANTITOXIN FLOCCULES

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DIPHTHERIA prophylaxis is receiving increasing attention in this country at the present time. An important aspect of the campaign is the problem of the maintenance of supplies of potent prophylactic agents by laboratories entrusted with their preparation. The experiences of one of these laboratories over a period of years in the development of methods of preparation of the different agents and in their biological assay by tests laid down in the regulations of the Therapeutic Substances Act (1931 and 1935) form the subject matter of this paper. Experimental work on the essential nature of toxoid antitoxin floccules—scientifically the most interesting of these agents—is described.

There are four prophylactic agents generally available for the immunisation of human beings against diphtheria.

F T (*formol toxoid*) is the culture filtrate of the Park Williams no. 8 strain of *C diphtheria*, the specific toxicity of which has been suppressed by means of formalin and heat. From this formol toxoid the other three agents can be prepared.

A P T (*alum-precipitated toxoid*) is a saline suspension of the well washed precipitate obtained by treating formol toxoid with potassium alum.

T A F (*toxoid antitoxin floccules*) is a saline suspension of the well washed flocculent precipitate obtained by treating formol toxoid with diphtheria antitoxin under defined conditions.

T A M (*toxoid antitoxin mixture*) is formol toxoid to which has been added a small amount of diphtheria antitoxin.

The following is a brief description of the methods of preparation and assay of *F.T.*, *A.P.T.* and *T.A.F.* It is unnecessary to deal with *T.A.M.* since it is doubtful if there is much justification for its continued use.

FORMOL TOXOID

This is made from culture filtrates of *C diphtheria* (the Park Williams no. 8 strain) by treating them with 0.3–0.4 per cent formalin and incubating at 38°–40° C until completely non-toxic.

Although this toxoid has proved valuable for the hyper-immunisation of horses, it is not always of sufficient potency for use in the human subject as judged by the standards laid down in the regulations of the Therapeutic Substances Act of 1931 and 1935. The following test, based on these regulations, has been used for assaying the immunising value of diphtheria prophylactics for clinical use.

An amount of the prophylactic equivalent to one tenth of a human dose is injected into more than ten white or cream guinea pigs on two occasions separated by an interval of not more than four weeks. The animals are tested for immunity to diphtheria toxin three weeks after the second injection of the agent, by the intracutaneous injection of one test dose of Schick toxin. If more than 25 per cent of the animals tested exhibit a positive Schick reaction, the agent is considered insufficiently potent for administration to man.

For the purpose of interpreting the data in this paper, therefore, it may be taken that where 75 per cent or more of the guinea pigs injected have become Schick negative, the prophylactic has been of sufficient potency to pass the test laid down in the regulations of the Therapeutic Substances Act.

*The immunising values of diphtheria formol toxoids
when tested on guinea pigs*

Whether or not a formol toxoid will pass the prescribed potency test in guinea pigs cannot in our experience, be predicted from its Lf value as determined by in vitro titration against diphtheria antitoxin. This is shown in table II, which includes the analyses and the immunising potencies of seven consecutive batches of F T.

TABLE II

*The analyses and immunising potencies of seven consecutive batches of
diphtheria formol toxoid*

No. of batch	Total solids (per cent)	pH	Lf units per c.c.	Immunising potencies after two injections at interval of four weeks	
				(A) of 0.1 c.c. of F T	(B) of 0.05 c.c. of A P T made from 1 l.
				Percentage of Schick negative animals three weeks after the second injection	
1	1.9	8.2	16.5	100	100
2	2.4	8.5	18.0	77	
3	2.7	7.6	16.5	88	100
4	2.4	8.6	24.0	60*	93
5	3.4	8.5	24.0	50	100
6	4.0	8.6	22.	20*	
7	2.9	8.7	18.0	64	93

Average volume of formol toxoid per batch = 100 l.

* Despite the failure of these batches of F T to pass the potency test in guinea pigs they gave antitoxic sera of titres up to 1400 (no. 4) and 1950 (no. 6) units per c.c. when used for the hyper-immunisation of horses.

Only two (nos. 1 and 3) out of the seven batches gave more than 75 per cent. Schick-negative animals after two spaced injections each of 0.1 c.c. The remaining five F.T., with substantially higher Lf values, all failed to pass the guinea-pig test. The wisdom of the English authorities in framing the regulations of the Therapeutic Substances Act so as to insist on animal tests rather than on in-vitro titrations, as in some countries, has been amply confirmed in the present studies. In France for instance, nos. 2, 4, 5, 6 and 7, with higher Lf values, would have been considered superior to nos. 1 and 3, whereas the animal tests emphasised their lower immunising values.

From five of the seven batches of formol-toxoid, including three which failed to pass the guinea-pig test, alum-precipitated toxoids were prepared by the method described below. All of these readily passed the test although the injected doses (0.05 c.c.) were only half those of the F.T. This emphasises what of course has long been established, that A.P.T. under similar conditions of injection is of superior immunising value to the formol-toxoid from which it is made.

Formol-toxoids 4 and 6 were used for the hyperimmunisation of horses. Despite their failure to pass the guinea-pig test, they produced antitoxic sera of titres up to 1400 (no. 4) and 1950 units (no. 6), per c.c.

ALUM-PRECIPITATED TOXOID

Sterile diphtheria toxoid, preferably of an Lf value of 15 or more units per c.c., is selected. Toxoid prepared from toxin which has a low total solids content, which flocculates rapidly with diphtheria antitoxin and which is rendered non-toxic after minimal treatment with formalin, probably provides the best type of product from which to prepare A.P.T. A pilot experiment for each batch of toxoid is carried out to determine the optimal conditions for precipitating the antigen with potassium alum. The latter is added in the form of a 5 or 10 per cent. sterile solution, the amount necessary to secure the maximum precipitation of Lf units being governed by the acidity of the alum solution. By increasing the acidity of its solution in water, the percentage of alum necessary can be kept low. The main batch of toxoid is subsequently precipitated under the determined optimal conditions. The white or slightly brown precipitate is washed two or three times by removing the supernatant liquid and replacing with sterile phenol-saline. If the use of alkaline saline is avoided and the sedimentation is carried out rapidly, the loss of Lf units at this stage is limited. The washed precipitate is suspended in sterile normal saline, preservative added and the volume adjusted for the time being to one-half or one-third that of the original formol-toxoid. This allows the necessary volume adjustments to be made after the product has been assayed in guinea-pigs.

The immunising potencies of 12 consecutive batches of A.P.T., with the analyses of the parent formol-toxoids, are shown in table III. The tests were carried out on samples where the volumes

were first adjusted to those of the original formol-toxoid, so that in each case 1 c.c. of A.P.T. was equivalent to 1 c.c. of the formol-toxoid from which it was made.

TABLE III

The immunising potencies of twelve consecutive batches of alum-precipitated toxoid, with analyses of the parent formol-toxoids

Formol toxoids				Alum-precipitated toxoids			
No of batch	Total solids (per cent)	pH	Lf units per c c	Immunising potencies after two injections at interval of four weeks			
				(A) 0.05 and 0.05 c c	(B) 0.025 and 0.025 c c	(C) 0.02 and 0.05 c c	(D) 0.01 and 0.03 c c
				Percentage of Schick negative animals three weeks after second injection			
1	1.9	8.2	16.5	100			..
2	2.7	7.6	16.5	100			.
3	2.4	9.0	19.5	93			..
4	2.3	8.8	21.0	100			.
5	2.6	8.7	19.5	100	82		.
6	3.4	8.5	24.0	100			.
7	2.9	8.7	18.0	93			.
8	2.6	8.7	15.0			100	64
9	2.6	8.7	15.0	100		92	50
10	3.2	7.6	12.0	89		71	.
11	3.0	8.6	13.5	100			20
12	2.8	8.7	15.0	100			38

Average volume of formol toxoid per batch = 75 l.

These results emphasise the outstanding immunising potencies of A.P.T. Only 3 out of 134 guinea-pigs failed to become Schick-negative after two spaced injections of 0.05 c.c. of the different batches (*i.e.* half the doses employed for the formol-toxoids shown in table II). For human immunisation, the regulations of the Therapeutic Substances Act would consider that two spaced injections each of 0.5 c.c., *i.e.* ten times the guinea-pig dose, of these A.P.T. would be adequate. In view of the desirability of limiting the size of the doses as far as possible, both from the point of view of economy and of minimising the chances of reactions, the effect of reducing the doses in the guinea-pig test was considered. In the only case where two injections each of 0.025 c.c. were given, 82 per cent. of the animals were rendered Schick-negative. In three cases where doses of 0.02 and 0.05 c.c. were given, 100, 92 and 71 per cent. respectively became Schick-negative. All four batches tested with doses of 0.01 and 0.03 c.c. failed to reach the 75 per cent. Schick-negative level demanded by the official test. It is possible from these results that most of the batches would have passed after two spaced injections of 0.025 and 0.05 c.c.; this would make the human doses 0.25 and 0.5 c.c. respectively.

TOXOID-ANTITOXIN FLOCCULES

Sterile diphtheria toxoid, preferably of Lf value 7.5 or more units per c.c., is selected. As with A.P.T., toxoid prepared from toxin which has a low total solids content, which flocculates rapidly with diphtheria antitoxin and which is rendered non-toxic after minimal treatment with formalin at 38°-40° C. probably provides the best material for making T.A.F. To this toxoid is added sterile diphtheria antitoxin, natural serum or (preferably) its concentrated globulins. The optimal conditions for doing this are not yet clearly defined. In practice a useful method seems to be to use very highly refined and concentrated globulins and to add a slight excess over that required for the most rapid flocculation with the particular toxoid. The resulting floccules can be heated to 80° C. (see below) without giving an undesirable granular type of product, and the final T.A.F. is of high although possibly not of maximal immunising potency. Further work remains to be done in this field. The flocculation of the toxoid-antitoxin mixtures can be carried out at 50° C. or at any lower temperature down to 5° C. There is some evidence that, with fast flocculating mixtures, temperatures between 5° and 20° C. are preferable.

The floccules are allowed to settle and are then washed repeatedly under sterile conditions with phenol-saline until all traces of colour have disappeared. They are then transferred to a pyrex glass flask, suspended in a small amount of sterile normal saline and heated to 80° C. for 30 minutes. The volume of the heated floccule suspension is adjusted with sterile normal saline to a volume equal to one-tenth that of the original formol-toxoid.

The immunising potencies of the floccule suspensions are tested on one, two or three batches each of more than ten guinea-pigs. Animals of the first batch are injected with two doses of 0.1 c.c., those of the second with two doses of 0.05 c.c., and those of the third with two doses of 0.025 c.c. The interval between the injections is one month and all the animals are Schick-tested three weeks after the second injection. From the results obtained, the dilution of T.A.F. for human use can be decided.

TABLE IV

The immunising potencies of twelve consecutive batches of T.A.F., with analyses of the parent formol-toxoids

Formol-toxoids				Toxoid-antitoxin floccules		
No. of batch	Total solids (per cent.)	pH	Lf units per c.c.	Immunising potencies after two injections at interval of four weeks		
				(A) of 0.1 c.c.	(B) of 0.05 c.c.	(C) of 0.025 c.c.
				Percentage of Schick-negative animals three weeks after the second injection		
1	2.1	8.6	16.5	93	71	...
2	2.6	7.8	16.5	100	60	...
3	2.6	8.9	15.0	92	64	...
4	2.2	8.2	7.5	95
5	2.4	8.2	13.5	100
6	2.5	8.5	19.5	67
7	2.5	8.6	10.5	77
8	2.3	8.7	15.0	100	100	100
9	2.6	8.7	16.5	92
10	2.5	8.7	19.5	100	82	...
11	3.4	8.2	16.5	100	100	...
12	2.5	8.8	15.0	100	100	100

Average volume of formol-toxoid per batch = 150 l.

The immunising potencies of twelve consecutive batches of T.A.F., with the analyses of the parent formol-toxoids, are shown in table IV.

With one exception (no. 6), all the batches passed the potency test at the 0.1 c.c. level; 4 out of the 7 tested passed at the 0.05 c.c. level and both of those tested at the 0.025 c.c. level. In practice this means that, assuming the human dose to be fixed at 1.0 c.c., then from 100 l. of formol-toxoid 10 l. of T.A.F. can be made if two doses each of 0.1 c.c. satisfy the guinea-pig test, 20 l. if two doses each of 0.05 c.c. are adequate, and 40 l. if two doses of 0.025 c.c. are sufficient. It is obvious that the immunising properties of T.A.F. can be increased at will merely by limiting the volume of saline in which the floccules are ultimately suspended. This must always be taken into consideration when comparing the immunising value of T.A.F. with that of other antigens.

The essential nature of toxoid-antitoxin floccules

Since the demonstration by Hartley in 1925 that the washed floccules from toxin-antitoxin mixtures were antigenic, enormous quantities of this type of product, now prepared from toxoid-antitoxin mixtures, have been used for the immunisation of human beings against diphtheria. Scientifically it is the most interesting of the agents available for the purpose, and opportunities have been taken during the past few years to study its essential nature and to effect possible improvements in its preparation and immunising potencies.

Early studies in this laboratory showed that the antigenic activity was not always confined to the floccules themselves but that, under certain conditions, the medium in which they were suspended became antigenic. If, for instance, suspensions of the floccules in neutral saline were filtered shortly after their preparation and before heating to 80° C., the filtrates showed, at the most, very slight antigenic activity. If suspensions of the unheated floccules were allowed to stand for long periods before filtering, the antigenic activity of the filtrates sometimes increased slightly. If, however, the suspensions were heated to 80° C., even for short periods, the floccules were partially dissolved and the antigenic activity of the filtrates increased suddenly and considerably.

The solubility of the floccules and the activity of these "antigenic filtrates" increased with the hydrogen-ion concentration at which the suspensions were heated. Maximum solubility and activity were usually reached at about pH 9.0. Above this the floccules remained in solution but the activity of the filtrates decreased and in highly alkaline solution was lost.

Ramon *et al.* (1931) and Povitzky (1932, 1935, 1939) have shown that if the floccules are freed from salt, suspended in distilled

water and heated to 80° C., they are almost completely soluble and the filtrate is antigenic. Ramon *et al.* visualised the toxoid-antitoxin complex—the floccules—as breaking down on heating, with destruction of the heat-labile antitoxin component and solution of the heat-stable antigen. If this conception were the correct one, it might reasonably be expected that the filtrates of the heated floccule solution would produce the typical flocculation phenomenon when mixed with diphtheria antitoxin.

If this essential confirmation could be obtained, these filtrates might be regarded as solutions of highly purified and concentrated antigen derived from the formol-toxoid. They could be standardised *in vitro* to a desired Lf unitage and this might lead to new developments in the preparation of prophylactics for human use. Unfortunately mixtures of the filtrates with different types of unconcentrated and concentrated diphtheria antitoxic sera examined over a period of years in this laboratory have almost invariably failed to exhibit the specific flocculation. Recently, however, it has been found that this reaction is produced when the filtrates are mixed with the “enzyme digest” type of highly concentrated serum similar to that described by Pope (1938, 1939). This observation has facilitated investigations into the preparation, essential nature and immunising properties of the antigenic filtrates. The following is a brief description of some of the results that have so far been obtained.

Optimal conditions for the preparation of an antigenic filtrate

The recovery of Lf units in the antigenic filtrate from the floccule solution after heating to 80° C. is dependent on the proportion of antitoxin used in the original toxoid-antitoxin mixtures. The following experiment illustrates this.

Equal volumes of formol-toxoid of Lf value 16.5 units per c.c. were treated with varying amounts of concentrated diphtheria antitoxin. The resulting floccules were centrifuged, suspended in equal volumes of distilled water and heated to 80° C. for 30 minutes. The solutions were filtered and their Lf unitage measured by *in-vitro* titration. The experiment was repeated with a second formol-toxoid of Lf value 18.0 units per c.c.

Fig. 1 shows the influence of the antitoxin content of the toxoid-antitoxin mixtures on the recovery of the Lf units in terms of the original toxoid.

The recovery of Lf units was complete only when the antitoxin was well in excess of that required to produce flocculation in the shortest time, *i.e.* at the so-called “flocculation point”. Recoveries were relatively low, both at this flocculation point and when the antitoxin was in considerable excess.

Marked purification of the diphtheria antigen is effected by this method. In the present series, for instance, the purified products contained nearly one hundred times as many Lf units per g. of dissolved matter as the original crude toxoid.

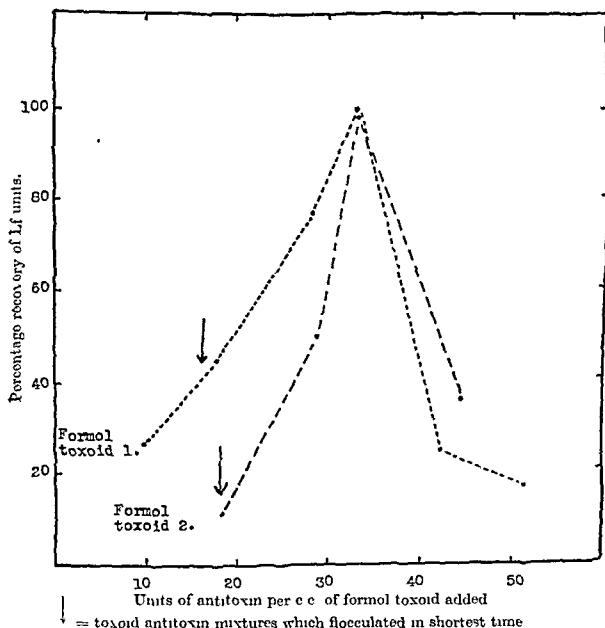


FIG. 1.—The influence of the antitoxin content of toxoid antitoxin mixtures on the recovery of Lf units in terms of the original toxoid

The essential nature of the filtrate

The effect of heating the floccules from toxoid-antitoxin mixtures to 80° C. in buffered saline suspensions at varying degrees of alkalinity was studied in the following experiment.

Equal volumes of the floccule suspensions in thin-walled pyrex glass bottles were adjusted to different hydrogen-ion concentrations and heated in a water-bath to 80° C. for 30 minutes. The solutions were then filtered and their content of Lf units measured by in-vitro titration. The results are shown in fig. 2.

The solubility of the floccules and the Lf values of the filtrates increased gradually with pH to a maximum at pH 9.0. At higher

hydrogen-ion concentrations the floccules remained in solution, but with increasing alkalinity the Lf activity diminished. The breaking down of the toxoid-antitoxin complex—constituting the floccules—into its antigen and antibody components, the gradual solution of the antigen on heating as the alkalinity increased to pH 9.0 and then its slow destruction as the alkalinity increased still further, are well illustrated in this experiment. The results are in line with the conception that solutions of heated floccules owe their antigenic activity to the presence of highly concentrated and purified diphtheria antigen derived from the crude formol-toxoid.

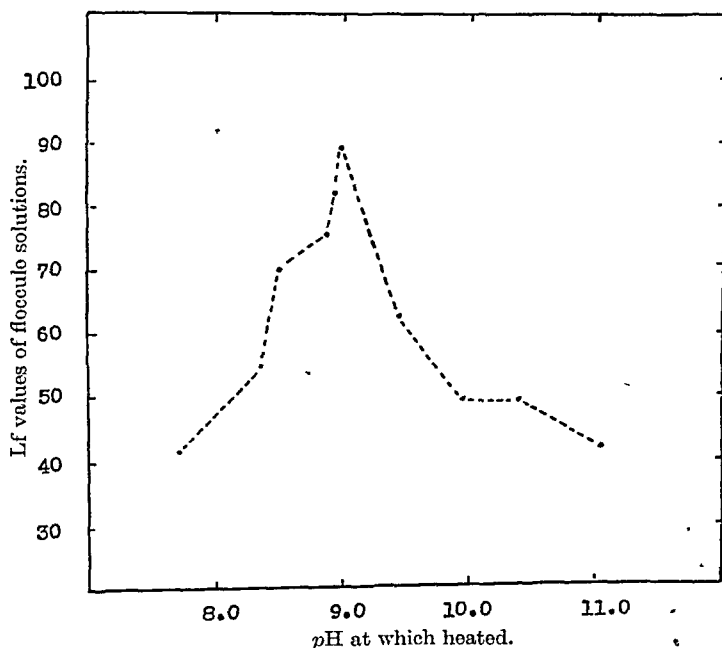


FIG. 2.—The effect of heating the floccules from toxoid-antitoxin mixtures to 80° C. in buffered saline suspensions at varying hydrogen-ion concentrations on the recovery of Lf units in the filtrates.

The immunising potencies of the filtrates

The highly concentrated and purified diphtheria antigen—as represented by the antigenic filtrates—can be obtained in the form of water-clear or slightly opalescent solutions which flocculate with antitoxin and contain mere traces of dissolved matter. These solutions can be standardised by in-vitro titration and in this way products of any desired potency can be obtained. An extensive series of animal tests would be necessary to determine whether the in-vitro measurements were any criterion of the immunising potency in guinea-pigs. Such measurements have been shown to be of no use for this purpose in the case of crude F.T. (see table II above).

It is possible, however, that the presence of impurities in this product may influence the intrinsic activity of the specific antigen and that a relationship may emerge when, as in the case of the antigenic filtrates, these impurities have been removed or reduced to a minimum. This point is being investigated but meanwhile it would seem that solutions of the purified antigen containing 50 Lf units per c.c. are sufficiently potent for human immunisation as judged by the test laid down in the regulations of the Therapeutic Substances Act.

Quantities of 0.1 c.c. of a solution of purified antigen containing 100 Lf units per c.c. were injected into 21 guinea-pigs on two occasions separated by an interval of three weeks. The animals were tested for immunity to diphtheria toxin three weeks after the second injection by the intracutaneous injection of one test dose of Schick toxin. The experiment was repeated with two other solutions, one containing 60 Lf units per c.c. (using 12 animals) and the other 50 Lf units (using 15 animals).

Three weeks after the second of the two injections, all 48 animals were found to be Schick-negative. It may be, of course, that solutions of these purified antigens containing less than 50 Lf units per c.c. will be adequate for the protection of human beings but extensive trials, both in animals and clinically, would be necessary to establish the minimum dose for this purpose.

DISCUSSION

The potencies of diphtheria prophylactics for human immunisation are safeguarded by the standards laid down in the regulations of the Therapeutic Substances Act. It has been shown that these standards can be readily maintained in the case of alum-precipitated toxoid and toxoid-antitoxin floccules made from the "low total solids" type of formol-toxoid described. They cannot however be maintained so readily for F.T. itself. Despite its high Lf value and invariable usefulness as sources of A.P.T. and T.A.F. and for the hyperimmunisation of horses, the untreated F.T. may, more often than not, fail to pass the guinea-pig test. Doubtless this is linked up with the rate of absorption of the antigen. With liquid agents of low total solids content, this may be rapid and sometimes too rapid for the antigen to exert its effect. When the absorption is slowed down, as in the case of A.P.T. made from the liquid agents, the antigenic effect is correspondingly increased. Broadly speaking, the ideal antigen is one which produces the maximum specific effect with the minimum of non-specific reactions. To attain the latter the amount of dissolved material in the prophylactic should be kept as low as possible consistent with the retention of reasonable Lf value. This has been accomplished in the present studies but only at the expense of limiting the number

hydrogen-ion concentrations the floccules remained in solution, but with increasing alkalinity the Lf activity diminished. The breaking down of the toxoid-antitoxin complex—constituting the floccules—into its antigen and antibody components, the gradual solution of the antigen on heating as the alkalinity increased to pH 9.0 and then its slow destruction as the alkalinity increased still further, are well illustrated in this experiment. The results are in line with the conception that solutions of heated floccules owe their antigenic activity to the presence of highly concentrated and purified diphtheria antigen derived from the crude formol-toxoid.

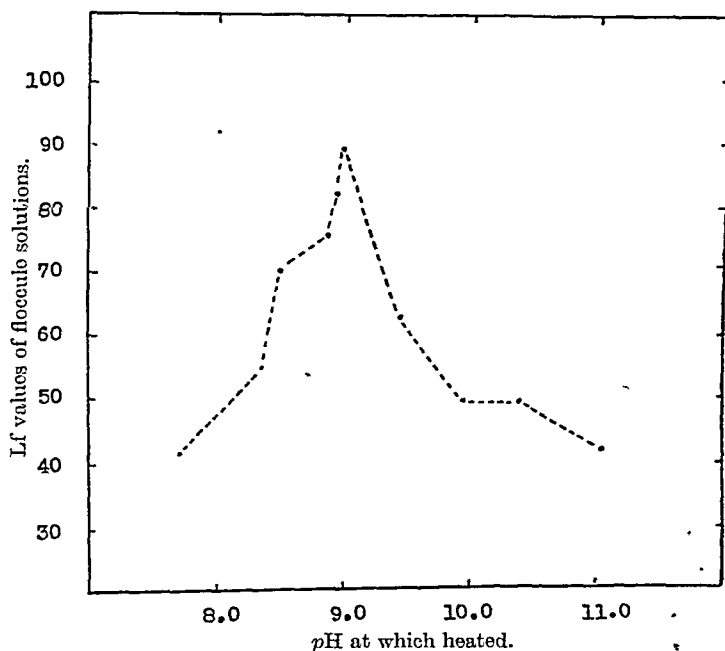


FIG. 2.—The effect of heating the floccules from toxoid-antitoxin mixtures to 80° C. in buffered saline suspensions at varying hydrogen-ion concentrations on the recovery of Lf units in the filtrates.

The immunising potencies of the filtrates

The highly concentrated and purified diphtheria antigen—as represented by the antigenic filtrates—can be obtained in the form of water-clear or slightly opalescent solutions which flocculate with antitoxin and contain mere traces of dissolved matter. These solutions can be standardised by in-vitro titration and in this way products of any desired potency can be obtained. An extensive series of animal tests would be necessary to determine whether the in-vitro measurements were any criterion of the immunising potency in guinea-pigs. Such measurements have been shown to be of no use for this purpose in the case of crude F.T. (see table II above).

solutions of this purified antigen are described. These solutions have a high immunising value in guinea-pigs and under certain conditions can be titrated *in vitro* against diphtheria antitoxin.

The authors are indebted to Mr H. F. Mannion and Mr J. Chapman for technical assistance.

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SOLITARY PLASMOCYTOMA OF BONE

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(PLATE III)

THE rarity of proven cases of solitary plasma-cell tumour of bone justifies the following report and review of previously recorded cases.

CASE REPORT

Clinical history. E S, a man of 45, was admitted to the Alfred Hospital on 3rd November 1939 under the diagnosis "neuro syphilis". He stated that in 1919 he had had a penile chancre and that nine months later paresis of the legs developed. Ever since then he had had foot drop, his feet dragged as he walked and he had constantly used crutches. At times he had noticed clonus of the feet on standing, and he tended to fall if his eyes were closed. He had also had some intermittent trouble with urination, namely difficulty in starting and slight incontinence if the bladder was overfull. Apart from the foregoing symptoms, he had remained well until three months before his admission, when he began to suffer with spasmodic attacks of pain in the back of the neck. This became more continuous and severe and at times radiated up over the back of the head. It was aggravated by movement of the neck. Examination on admission disclosed the following noteworthy physical abnormalities. All deep reflexes were exaggerated, the plantar reflexes were extensor in type and the superficial abdominal reflexes were absent. Knee and ankle clonus were present and the legs spastic but with fair power. All kinds of sensation in the lower limbs showed some impairment. There was no neck rigidity. Near the base of the glans penis there was a scarred area with some pigmentation. The blood Wassermann was negative. Two days after admission pyrexia up to 100.6° F appeared. On 7th November total paralysis of the legs and arms developed and the patient died on the following day.

Autopsy findings. In the thoracic and abdominal viscera the only noteworthy abnormalities were extensive bronchopneumonia of the left lung with early empyema, purulent urine and general congestion of the viscera. The head was unusually movable on the spinal column and, on looking down through the foramen magnum after the brain had been removed, it was noted that the skull and atlas could be displaced forwards, backwards or sideways on the subjacent vertebræ, with almost total obliteration of the spinal canal. As a result the lower part of the medulla oblongata and the upper part of the spinal cord had been crushed. A lateral

radiogram (fig. 1) revealed an extensive destructive lesion of the entire second cervical vertebra and on dissection the body, dens, and neural arch were all found to be largely replaced by soft friable hæmorrhagic tissue. Sections of the remaining vertebræ, the skull, ribs, sternum and shaft of the right femur failed to reveal any other bony lesion.

Histology. Bony tissue was very scanty, being represented only by occasional isolated trabeculæ in the substance of the growth and by an incomplete shell of thinned cortex at parts of its periphery. Where no peripheral bone was present, the growth infiltrated the surrounding ligaments and muscles. Areas of hæmorrhage and of fibrosis were plentiful in the tumour, associated in places with collections of foam cells and pigment-laden phagocytes, while occasional foreign-body giant cells were present near areas of hæmorrhage. The essential tumour tissue (figs. 2 and 3) consisted of large or small areas of typical plasma cells averaging about 10-12 μ in diameter, each with an eccentric nucleus with a characteristic chromatin pattern and abundant cytoplasm sometimes visibly differentiated into two zones. A few large multinucleated cells of the same type were present. Mitotic figures were very scanty.

DISCUSSION

Interpretation of the case reported

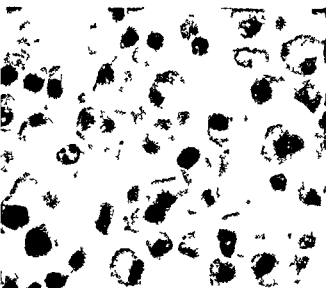
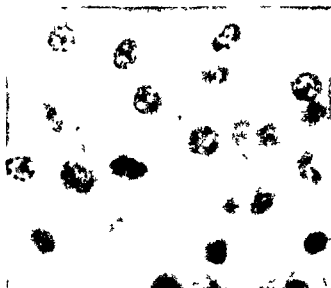
The case was clearly one of almost total plasmocytomatous replacement of the second cervical vertebra without demonstrable involvement of any other part of the skeleton. The nature of the disease was not suspected during life and there is no record of the results of tests for Bence-Jones proteose in the urine. Although radiograms of the entire skeleton were not obtained, the deliberate search for multiple skeletal lesions made *post mortem* leaves little doubt that the vertebral growth was a solitary one.

The case is of interest as regards the possible duration of the tumour and its relationship to the symptoms from which the patient suffered. It is clear that the finally complete paraplegia was due to the tumour, and in retrospect it seems highly probable that the spastic paresis of the legs dating from 1919 was also due to mild compression of the cord by the tumour. Although the history suggested a diagnosis of neuro-syphilis, the negative Wassermann test and the absence of any post-mortem findings suggestive of nervous syphilis serve to eliminate this possibility. The long duration of the parietic symptoms—20 years—is not incompatible with the view that the tumour was the causative agent, since several other of the recorded cases of solitary plasma-cell tumours reviewed below were also known to have been present for many years. It seems very probable then that the tumour had indeed been present

SOLITARY PLASMOCYTOMA OF BONE



FIG. 1—Lateral radiogram of cervical spine after post mortem removal

FIG. 2—Photomicrograph of paraffin section of tumour tissue stained by hematoxylin and eosin $\times 1000$ FIG. 3—Section of tumour tissue stained by toluidine blue showing the typical characters of the plasma cells $\times 1200$



for this long period, and had all along been the cause of the paresis, which finally became complete and ended fatally. A possible causative relationship of previous syphilis to the plasma cell growth is too indefinite to deserve more than passing mention. In previously reported cases of plasma cell tumour, syphilis has not been recorded as an antecedent.

Review of previous records

Solitary plasma cell tumours of bone are rare, less than a score of cases in which this diagnosis can be accepted have been reported. The lesions of myelomatosis often consist of plasma cells, and clearly, before making a diagnosis of solitary plasma cell tumour there must be strong evidence that the case in question is not one of myelomatosis with one tumour clinically conspicuous at an early stage of the disease. Acceptable clinical evidence is afforded if complete, competent and, if necessary, repeated radiographic examination of the entire skeleton fails to disclose more than the one lesion, and if a long period elapses after initial diagnosis and treatment without signs of the development of further lesions or the appearance of Bence Jones proteosuria. In fatal cases, proof of the solitary nature of the tumour is afforded by adequate post mortem examination. In the following review an attempt will be made to distinguish cases for which there is good evidence of the solitary nature of the tumour from those which fail to meet these requirements.

Probably the first clear case of solitary plasma cell tumour of bone to be recorded was that of Shaw (1923). The patient was a man of 29 with a pathological fracture of the middle of the shaft of the humerus. Radiograms of the rest of the skeleton failed to reveal any other lesions. Tests for Bence Jones proteose were negative and, according to Cutler *et al* (1936), the patient was alive without signs of recurrence nine years later.

In 1924 Walthard reported a case of plasma cell tumour of the seventh cervical and first thoracic vertebrae with compression of the spinal cord in a man of 55. Symptoms had been present for ten months prior to operation. The patient died following laminectomy and at autopsy no lesions were found in any other part of the skeleton.

Zdansky (1927, quoted by Cutler *et al*) described a case of plasma cell tumour in the upper third of the femur in a woman of 68 who had had pain for one year and who died soon after a pathological fracture. Radiograms of other parts of the skeleton and post mortem examination failed to reveal any other tumours.

A plasma cell tumour of the neck of the femur in a woman of 56 was reported by Martin *et al* (1928). Bence Jones proteosuria was not present. The patient remained well over two years after amputation.

In 1929-30 Rogers reported a case of plasma cell tumour of the shaft of the femur with pathological fracture in a man of 34. Radiograms failed to reveal lesions in any other bones and the urine did not contain Bence Jones proteose. According to a personal letter from Professor Hey Groves to Cutler *et al*, the patient remained well and without any signs of generalisation four years after amputation.

this tumour has been mistaken for endosteal sarcoma and for giant-cell tumour, and it can also readily simulate the appearances of a metastatic growth.

Prognosis: the possible risk of generalisation

Regarding prognosis, we must ask what risk is there of a plasmocytoma initially solitary according to the above criteria being succeeded by generalised myelomatosis. Certain recorded cases require comment in this respect. In 1934 Peyton reported the case of a man of 49 who, following symptoms of one year's duration, was found to have a plasma-cell growth of the sixth dorsal vertebra. Radiograms of the rest of the skeleton were not obtained at this time and the urine was not tested for proteose. After operation and radiation treatment he improved, and 18 months later general radiograms showed no other tumours. At this time, however, proteosuria was present, and the patient died with generalised disease of the skeleton two years later (Cutler *et al.*). In this case there must remain some doubt as to the solitary nature of the growth from the beginning. At the time operation was performed no evidence was obtained that the tumour was indeed solitary, and the later failure to reveal other bone lesions by radiographic examination at a time when proteosuria was present appears contradictory. A second case reported by Peyton and included as no. 13 in the review of Cutler *et al.* was clearly generalised from the outset. In Bloodgood's case of plasmocytoma of the clavicle, reviewed by Cutler *et al.* as no. 11, proteosuria was discovered soon after operation and a rib lesion was detected radiographically two months later. Clearly this case also must be regarded as one of myelomatosis from the beginning, with a clinically precocious tumour in the clavicle. In case 14 of Cutler *et al.*—one of plasma-cell growth in the femur of a man of 56—radiograms of other bones failed to reveal other lesions, and proteosuria was not present; but death with generalised disease and proteosuria occurred less than three years later. In this case the interval between the original diagnosis and the development of demonstrable generalisation is unknown and there must remain some suspicion that the case was all along of generalised nature with early prominence of the femoral tumour. In case 15 of Cutler *et al.*, an interval of only 9 months elapsed between initial diagnosis and the discovery of gross generalisation; and their cases 19 and 20 also were clearly generalised from the outset. In a case of vertebral plasmocytoma described by Espersen (1934), the patient remained apparently well for one year following operation and radiotherapy, but radiograms then showed involvement of two other vertebrae and a rib.

In none of these cases then is there completely convincing evidence that the tumour first diagnosed was indeed solitary. It

is important to realise that in cases of myelomatosis small scattered foci may easily escape radiographic detection and that an initially prominent tumour might easily appear to be a solitary one until others become unmistakable in radiograms, perhaps only some months later. Since radiograms are not often repeated at frequent intervals, discovery of generalisation by this means must often be delayed for weeks or months. Bence-Jones proteosuria must be looked upon as evidence of extensive involvement of bone marrow in spite of negative radiographic findings.

To distinguish between a clinically and radiologically precocious lesion of multiple myeloma and a truly solitary plasmocytoma it is essential that the following rules, suggested earlier in this paper, should be adhered to :—(a) highly competent radiographic examination of the entire skeleton, including the skull, should be obtained, and if the lesion then appears to be solitary this examination should be repeated in 6 months time, and again in a year ; (b) the urine should be examined for Bence-Jones proteose at frequent intervals during this period. If thorough radiographic examination one year from the date of tentative diagnosis is unequivocally negative and proteosuria has been regularly absent, it is improbable that signs of generalised myelomatosis will appear later, and the tumour may be diagnosed with reasonable certainty as a solitary one.

The pathological relationship of solitary and generalised plasma-cell tumours of bone

The question arises, are solitary plasmocytoma and multiple plasma-cell myeloma two unrelated pathological entities or are they variants of a single tumour type ? The number of thoroughly studied cases of apparently solitary tumours is as yet too small to permit of certainty, but the following facts are significant.

(a) *A plasmocytoma of bone may exist as a solitary growth for a very long period.* In several of the recorded cases there has been clinical evidence of the presence of the tumour for a number of years prior to its discovery or treatment, e.g. in the case reported in this paper for probably 20 years. Other tumours, which have caused few or no symptoms prior to pathological fracture or other complication, have nevertheless had the bulk and other characters of well established growths of considerable age. The mitotic activity of plasmocytomas is not great and bulky tumours (as many of them are) must have been present for long periods prior to the first appearance of symptoms. Again in some cases the patients have been kept under observation for many years after initial diagnosis and treatment and no further lesions have appeared.

(b) *Cases of multiple myeloma, if adequately investigated, usually have demonstrable multiple lesions early in the course of the disease.*

The great majority of the cases conform with the classical picture of extensive and progressive affection of bone marrow, with multiple fractures, leading to a cachectic state and death within a few years. Initial simulation of a solitary lesion for a long period is very unusual, and, as our review above shows (p. 82), even in those rare cases in which a supposedly solitary tumour has been followed later by generalised disease, the evidence that the tumour was initially solitary is often inconclusive.

On the one hand, then, it is certain that a plasmocytoma of bone may remain solitary for many years, and, on the other, that the generalised character of myelomatosis is usually obvious from an early stage of the disease. This sharp contrast strongly suggests the existence of two distinct types of growth. Moreover, contrary to the contention of Cutler *et al.* that generalisation takes place by metastasis, most competent pathologists share the view that myelomatosis is in its nature a generalised or systemic disease of the marrow and that metastasis plays no part in its development. If we believe that generalisation occurs by metastasis from an initial solitary focus we will look upon solitary plasmocytoma and myelomatosis as essentially related. If, however, we adhere to the much more probable view that myelomatosis is a system disease, then solitary plasmocytoma must be looked upon as a separate and distinct entity.

SUMMARY

1. A case is described in which autopsy revealed fatal compression of the spinal cord due to a solitary plasmocytoma of the second cervical vertebra. The history suggested that the tumour had been present for at least 20 years.

2. Previously reported examples of solitary plasmocytoma of bone are reviewed. Thirteen cases are accepted as certainly solitary ; in a number of other possible cases there was some doubt as to the solitary nature of the growth.

3. Solitary plasmocytoma occurs chiefly in middle age and men are affected more often than women. Pathological fracture is frequent. Radiograms show a central rarefying lesion which has often been mistaken for endosteal sarcoma or for giant-cell tumour. Diagnosis requires (a) exclusion of the possibility of generalised myelomatosis and (b) exploratory operation and microscopical identification of the tissue.

4. Solitary plasmocytoma is probably a distinct entity and not merely an early localised stage of myelomatosis. With adequate surgical or radiation treatment, the prognosis is good.

I am indebted to Dr J. F. Chambers for access to the clinical records of the case reported.

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THE TOXINS PRODUCED BY *CLOSTRIDIUM WELCHII* IN A SIMPLE MEDIUM

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THE experiments described below were designed in an attempt to produce the toxins of the *Cl. welchii* group of organisms in a simple medium containing no animal tissue.

Since the demonstration by Wilsdon (1931) and Glenny *et al.* (1933) of the multiplicity of toxins produced by the *Cl. welchii* group, several workers have studied the influence of different factors on the production of toxins. The subject was reviewed in detail by Dalling and Ross (1938), who used meat infusion broth containing peptone and the minced horse muscle used in the preparation of the broth. They showed that the production of toxin by the different types of *Cl. welchii* was influenced not only by the period of incubation but also by the proportion of meat particles present and by the pH of the medium. In this Institute, despite the employment of an identical technique and a similar pH (7.6-7.8) in the preparation of each batch of meat broth medium, great variation in individual batches, as gauged by the toxin production of *Cl. welchii* type B, led us to investigate the possibilities of a simplified medium. The classification employed throughout this paper is that devised by Wilsdon (1931, 1932-33). Subsequent literature on this subject has been reviewed in detail by McCoy and McClung (1938).

Several attempts to obtain growth in media containing only salts and glucose were unsuccessful. Finally, by the addition of casein and glucose to a solution described by Scott (1930) containing only salts and peptone, a vigorous growth of *Cl. welchii* was obtained without the use of special anaerobic methods.

METHODS

Medium. The composition of the medium was as follows.

"Bacto" peptone	7.5 g.
Casein	7.5 "
Potassium bicarbonate (KHCO_3)	0.25 "
Ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$)	0.3 "
Dipotassium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	0.85 "
Iron ammonium citrate	1.0 "
Glucose	5.0 "
Tap water	500 ml.

Sterilisation was at first effected by steaming for one hour on each of three successive days, but it was subsequently found that twenty minutes at 15 lb. in the autoclave had no adverse effect and this method was adopted.

Cultures. The following strains of *Cl. welchii* were used.

Type A. N.C.T.C. 26, obtained from Professor Dalling in 1935, and B. 14/3, isolated from the intestinal contents of a normal bovine in 1939 (Taylor and Gordon, 1940).

Type B. These were isolated in this Institute from natural cases of lamb dysentery, 36/8 in 1936 and 4228 and 4229 in 1940. In a suitable medium all were capable of producing type D toxin as well as type C.

Type C. 993, 878 and 11 A (Montgomery and Rowlands, 1936), and W 49, received from Dr R. F. Montgomery in 1937.

Type D. 35/4, isolated from the small intestine of a horse in 1935, and 36/2 and 4265, isolated from cases of "pulpy kidney disease" of lambs in 1936 and 1940 respectively.

Antitoxins. Type A (N.C.T.C. 26), type B (Adra) and type D (35/4) have already been described by Taylor (1940) and Taylor and Gordon (1940), while supplies of type C (R 3589) were available through the courtesy of Dr O'Brien of the Wellcome Research Laboratories.

Mixtures of toxin and antitoxin were kept at laboratory temperature for at least 30 minutes before inoculation into mice of 20-24 g. Culture fluid for these tests was obtained by centrifuging whole cultures for 30 minutes at 3000 *r.p.m.* Dry toxin was prepared from British Berkefeld-candled culture fluid by precipitation with ammonium sulphate and drying *in vacuo* over CaCl_2 . For use, 100 mg. of dry toxin were dissolved in 5.0 c.c. of normal saline.

RESULTS

Every strain of *Cl. welchii* examined grew readily in the medium, which was clotted and disrupted by gas as in the "stormy clot" fermentation of milk medium. The organisms died out rapidly at 37° C.; subcultures could be obtained after 3 but not after 5 days at that temperature.

Daily subcultivation of representative strains of all four types of *Cl. welchii* over a period of 105 days resulted in no change in the type or toxicity of the strains on their transference to meat broth medium.

Toxin production

Type A. Both cultures grew vigorously, but neither produced readily detectable toxin. One-half c.c. of culture fluid was non-lethal to mice, but two guinea-pigs inoculated intramuscularly with 0.5 and 0.1 c.c. respectively of an actively growing culture of N.C.T.C. 26 died overnight with typical gas gangrene. In meat broth medium, the M.L.D. of each strain for mice was 0.025 c.c. of culture fluid.

Type B. The M.L.D. for mice of culture fluid of each strain remained fairly constant at 0.005 c.c., while in meat broth medium it varied from 0.001 to 0.005 c.c. Toxin-antitoxin tests showed the toxin present to be type C, as even after four days' incubation it was still neutralisable by type C as well as by type B antitoxin.

Type C. With two of the strains—993 and 828—the M.L.D. of the culture fluid from meat broth medium was 0.05 and 0.01 c.c. respectively, whereas 0.1 c.c. of that from the special medium was

non lethal to mice The two remaining strains—11 A and W 49—produced in meat broth a toxin with a M L D of 0 001 and 0 0005 c c respectively, in the special medium the culture fluid was lethal in doses of 0 01 and 0 005 c c The toxin present was shown to be type C by toxin antitoxin tests

Type D Production of toxin by these organisms was uncertain Strain 35/4 was invariably non toxic in a dose of 0 5 c c of culture fluid, but 36/2 and 4265 were occasionally lethal in similar amounts and the toxin present was shown to be type D On these occasions, toxin production did not appear to be greatly influenced by the period of incubation, while culture fluid from all three strains in meat broth medium incubated for four days was lethal to mice in doses of 0 0025 c c

These results were repeated and confirmed on numerous occasions

Toxin antitoxin reactions

These were determined by the intravenous injection of mice

Dry toxins were precipitated from cultures grown in the special medium, and, with these, antitoxins were prepared in rabbits These are designated as in table I, in which the prefix "S" denotes a toxin prepared in the special medium and a final "A" in the prefix denotes an antitoxin, the intervening letter indicating the type The table also shows the minimal hæmolytic dose of each dry toxin, namely the amount required to produce 50 per cent hæmolysis in 1 0 c c of a 6 per cent suspension of sheep red blood cells

TABLE I

Designations and activity of toxins and antitoxins used

Culture	Toxin no	M L D for mice	M H D	Antitoxin no
Type A N C T C 26	SA 27340	Non toxic	10 mg	S A A 151040
Type B 36/8	SB 26140	0 02 mg	>20 ,	S B A 24740
Type C W 49	SC 20240	0 1 „	>20 ,	S C A 25740
Type D 36/2	SD 28340	Non toxic	>20 „	S D A 141040

Dry toxins precipitated from meat broth culture fluid were also used The type A, N C T C 26 (toxin A 91137), type C, W 49 (toxin C 11138) and type D, 35/4 (toxin D 23636) toxins have already been described by Gordon *et al* (1940), the M L D for mice were 0 6, 0 02 and 0 004 mg respectively The type B toxin (B 18/18636) was prepared from a culture of several strains incubated for 18 hours, the M L D for mice was 0 02 mg

Table II shows the results of toxin-antitoxin tests between sera and toxins prepared both in meat broth and in the special medium. Many of the figures given are approximate, having been calculated from the amount of antitoxin required to neutralise 10 or 100 mouse lethal doses, and are presented thus merely for the sake of uniformity in comparison. Repetition of these tests gave a similar result on each occasion.

TABLE II

Number of mouse M.L.D. of each toxin neutralised by 0.1 c.c. of each antitoxin

Antitoxin	Toxin					
	Type A A 91137	Type B SB 26140	Type B B 18/18636	Type C SC 20240	Type C C 11138	Type D D 23636
Type A S.A.A. 151040 . . .	0	0	0	0	0	0
Type A N.C.T.C. 26 . . .	250	0	0	0	0	0
Type B S.B.A. 24740 . . .	2	250	250	250	250	15
Type B Adra	20	3300	3300	3300	3300	330
Type C S.C.A. 25740 . . .	3	250	250	250	250	0
Type C R. 3589	10	1000	1000	1000	1000	0
Type D S.D.A. 141040 . . .	10	0	0	0	0	10
Type D 35/4	100	0	0	0	0	1100

Culture fluids SA 27340 (type A) and SD 28340 (type D) were also tested but were non-lethal for mice.

It is shown in table II that of the two non-toxic precipitates obtained from cultures on the simple medium, one, type A, S.A. 27340, was non-antigenic in that its "antitoxin", S.A.A. 151040, was incapable of neutralising 1 M.L.D. of a true type A toxin (A 91137), while the other, type D, S.D. 28340, stimulated the production of true type D antitoxin, S.D.A. 141040, which was capable of neutralising both type A and type D toxin. Type B toxin S.B. 26140, which by the "mouse intravenous" test was that of type C, produced a true type B antitoxin capable of neutralising the toxins of all four types, while type C toxin S.C. 20240 also gave rise to a true type C antitoxin.

The effect of enzymes on toxin production

Bosworth and Glover (1934-35) showed that the apparent toxicity of type D toxin was greatly increased by the addition of trypsin, and more recently Dalling and Ross (1938) have found that trypsin, when added to medium in which type B is grown, permits of the production of type D toxin only.

The addition of trypsin to the special medium was also found to affect toxin production by types B and D. If trypsin were added before inoculation and incubation with type B strains, only type D toxin developed. If after overnight incubation of type B strains, when only type C toxin could be demonstrated, trypsin were added and incubation continued overnight, type D toxin was formed in considerable amount, while the type C toxin was no longer detectable.

In the special medium the culture fluid from *Cl. welchii* type D was only occasionally toxic in a dose of 0.5 c.c. After the addition of trypsin to the medium, however, cultures were regularly lethal to mice in a dose of 0.005 c.c. This effect was produced after overnight incubation when the trypsin was added to the medium at the time of inoculation, but when incubation was prolonged for four days the toxicity was greatly decreased.

A similar but less marked effect was also obtained by the use of pepsin, but may in fact have been due to impurity.

The addition of trypsin to a solution of dry type B toxin SB 26140 resulted in its being converted to type D; similarly, trypsin converted the ordinarily non-lethal D precipitate SD 28340 into type D toxin.

The effect of trypsin as noted above was repeated and confirmed on several occasions.

The influence of pH upon toxin production

It was found by Dalling and Ross that the pH of cultures of both types A and D in meat broth medium fell from 7.5 to 6.5 or 6.0, but after a few days that of type D cultures rose towards the original level. They also found that type D toxin was not formed at pH 5.0 although the toxin production of types B and C was apparently not affected by this degree of acidity.

During incubation with any of the four types of *Cl. welchii*, the pH of the special medium fell rapidly from 7.4-7.6 to 4.8-5.2, and it was thought that this fall might conceivably be the factor inhibiting the production of types A and D toxin. Accordingly, the following experiments were performed. Four pairs of flasks, each flask containing 300 c.c. of the special medium, were steamed for 40 minutes and cooled. Each pair was then inoculated with approximately 5 c.c. of actively growing culture of one of each of

TABLE III

Effect of pH on toxin production

Culture	Flask	4 hours			8 hours			12 hours			24 hours			48 hours			4 days		
		pH	M.L.D.	Toxin	pH	M.L.D.	Toxin	pH	M.L.D.	Toxin	pH	M.L.D.	Toxin	pH	M.L.D.	Toxin	pH	M.L.D.	Toxin
A N.C.T.C. 26	Ad.	5.8	>0.5	...	6.4	>0.5	...	7.0	>0.5	...	7.6	>0.5	...	7.6	>0.5
	Co.	5.8	>0.5	...	5.4	>0.5	...	5.0	>0.5	...	5.0	>0.5	...	5.0	>0.5
B 4229	Ad.	5.8	0.005	C	5.8	0.001	C	6.8	0.01	B	7.2	0.0075	D	7.4	0.0075	D	7.4	0.0075	D
	Co.	5.8	0.005	C	5.4	0.005	C	5.2	0.005	C	5.0	0.005	C	4.9	0.005	C	4.9	0.005	C
C W 49	Ad.	5.6	0.005	C	5.8	0.00025	C	6.2	0.00025	C	7.2	0.0005	C	7.6	0.0005	C
	Co.	5.6	0.005	C	5.4	0.005	C	5.0	0.0025	C	5.0	0.0025	C	4.8	0.0025	C
D 4265	Ad.	5.8	>0.5	...	5.8	0.25	...	6.0	0.075	...	7.6	0.05	D	7.5	0.05	D	7.5	0.01	D
	Co.	5.8	>0.5	...	5.4	>0.5	...	5.2	>0.5	...	5.0	>0.5	...	5.0	>0.5	...	5.0	>0.5	...

Ad. = pH adjusted as described in text.

Co. = unadjusted during growth (control).

M.L.D. expressed as c.c. of culture fluid.

the four types of *Cl. welchii* and incubated at 37° C. After 4, 8, 12, 24, 48 and 96 hours' incubation, samples were withdrawn from each flask for examination. After the withdrawal of each sample the pH of one of each pair was raised to 7.6-7.8 by the addition of NaOH. The results obtained are recorded in table III. This experiment was carried out in duplicate with a similar result.

These experiments show that type A, even during active growth, produced no toxin detectable by the intravenous inoculation of mice. After eight hours' incubation type B is relatively more toxic in adjusted medium, but it is less toxic after twelve hours, and although on further incubation the toxicity increases, it does not attain the level of that of the unadjusted (control) medium. The toxin formed in the control medium, however (tested by the mouse intravenous method only), is type C, while in adjusted medium it is at first type C, then for a short period type B, then after twenty-four hours type D. The toxin of type C is also developed more rapidly and in considerably greater quantity in adjusted medium than in the control. Type D produces no detectable toxin in the control medium, but in the adjusted medium a gradually increasing quantity of toxin becomes demonstrable and reaches a maximum after four days' incubation.

DISCUSSION

In the simple medium described above all four types of *Cl. welchii* grow readily, but the methods employed—intravenous inoculation of mice, tests for the presence of hæmolysin and preparation of antitoxin—have been unable to detect the presence of any appreciable quantity of type A toxin.

Type B produces a potent toxin which, when tested by the "mouse intravenous" method alone, is apparently type C, its antitoxin, however, is shown to be that of a true type B in that it neutralises the toxins of types A, C and D (table II). That type D antigen is present in cultures of type B has been further shown by the addition of trypsin to the medium or by the artificial maintenance of the pH of the cultures, both of which methods permit of the formation of readily detectable type D toxin.

Toxin production by type C is also affected by the maintenance of pH, the toxin formed being as much as ten times greater in an adjusted medium.

Cultures of type D in the medium produce no toxin detectable by the intravenous inoculation of mice, but type D antigen is evidently present, as antitoxin prepared against dried material precipitated from culture fluid neutralises the toxins of types A and D (table II). Cultures are affected similarly to those of type B by the addition of trypsin or by the artificial maintenance of the pH, as both these procedures permit the detection of type D toxin.

There is therefore some appreciable difference between the toxigenic requirements of *Cl. welchii* type A and those of the other members of the group. Some factor, present in meat broth but not in the simple medium and not associated either with the enzyme trypsin or with the hydrogen ion concentration of the medium, is necessary for the formation of toxin by type A.

SUMMARY

A simple medium consisting only of peptone, casein, salts and water is described in which organisms of the *Cl. welchii* group grow readily.

No toxin lethal for mice is produced by types A and D in this medium, but potent toxin is formed by types B and C.

The addition of trypsin permits of the production of type D toxin by types B and D. A similar effect is obtained by the artificial maintenance of the hydrogen ion concentration of cultures at or about pH 7.6 during growth.

The significance of these findings is discussed.

We are indebted to Dr R. A. O'Brien for antitoxin, to Professor T. Dalling and Dr R. F. Montgomerie for cultures and to the director of this Institute, Dr J. Russell Greig, for his continued interest in this work.

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IN-VITRO PRODUCTION OF TOXIN FROM STRAINS OF *CL. WELCHII* RECENTLY ISOLATED FROM WAR WOUNDS AND AIR RAID CASUALTIES

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THE occurrence of *Cl welchii* in wounds in which there are no symptoms of gas gangrene has often been observed. This has led to the assumption that many *Cl welchii* strains are atoxic. It seemed therefore opportune to investigate this matter with the material afforded by the present war. It should be understood that the aim of this work was to correlate the in vitro capacity of the strain to produce soluble toxin with its in vivo pathogenicity as illustrated by the general nature of the case from which it was isolated. The potency of the fluids produced was viewed therefore, not from the quite artificially exacting standard of the serum maker, which has too long dominated our understanding of *Cl welchii* toxin, but from the standpoint of measurable toxicity in relation to the recent clinical behaviour of the organism.

The study of *Cl welchii* toxin has hitherto been carried out with a very small number of strains chosen in all cases only for their high toxin producing capacity and the methods have been adapted to these particular strains. It should be kept in mind that the species *Cl welchii* (type A, gas gangrene) includes a group of organisms whose identification is based on similarity in morphology and gross cultural reactions and on a broad correspondence in the toxin complex produced, while the somatic bacterial antigen appears to be so variable as to be strain specific (Henderson, 1940).

MATERIAL AND METHODS

Twenty six strains have been investigated and we are much indebted to Professors A. A. Miles, J. McIntosh and A. Fleming, Drs E. T. C. Spooner, J. Murray and J. C. Colbeck of the Emergency Medical Service and Dr Lucy Wills of the Royal Free Hospital, who isolated the organisms from their cases and provided the clinical histories. This very valuable co-operation formed the basis of the investigation.

It will be seen in the sequel that the material came from cases of very diverse types and a clinical arrangement of the strains into three groups in accordance with their origin was made.

There is therefore some appreciable difference between the toxigenic requirements of *Cl. welchii* type A and those of the other members of the group. Some factor, present in meat broth but not in the simple medium and not associated either with the enzyme trypsin or with the hydrogen ion concentration of the medium, is necessary for the formation of toxin by type A.

SUMMARY

A simple medium consisting only of peptone, casein, salts and water is described in which organisms of the *Cl. welchii* group grow readily.

No toxin lethal for mice is produced by types A and D in this medium, but potent toxin is formed by types B and C.

The addition of trypsin permits of the production of type D toxin by types B and D. A similar effect is obtained by the artificial maintenance of the hydrogen ion concentration of cultures at or about pH 7.6 during growth.

The significance of these findings is discussed.

We are indebted to Dr R. A. O'Brien for antitoxin, to Professor T. Dalling and Dr R. F. Montgomerie for cultures and to the director of this Institute, Dr J. Russell Greig, for his continued interest in this work.

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On testing one particular arrangement of the ingredients, it was soon observed that newly isolated strains reacted in different ways. Not only did the lethal effect (toxicity) for mice differ, but the degree of acidity produced in the medium by the fermentation of the available sugar also varied. By observing the *pH* values, particularly at 3-4 hours, which appeared usually to coincide with the peak of the fermentation and the evolution of gas, and at 6-7 hours, when the maximum toxin was developed, it became evident that strains produced their best toxin at certain *pH* values. More precisely stated, the development of toxin was observed to be correlated with growth which produced a certain *pH* cycle in the medium. The changes in *pH* value during growth were looked upon as indications of the nature of the growth cycle in the strain under observation and no absolute importance apart from this was attached to them, nor was the acidity considered to be the only factor controlling the production of toxin.

Some of these points are illustrated in table I, where it is seen that the first four strains, grown in the same medium with the same high sugar content of 0.6 per cent, produced very different *pH* values—LP, 4.8, JCC, 6.1. This table also shows that the *pH* value was

TABLE I

Variation in toxin production by different strains of Cl welchii

Strain	Medium			<i>pH</i> values			Mouse M L D (c c)
	Peptone (per cent)	Glucose (per cent)	Serum (per cent)	Initial	3½ hrs	~ hrs	
LP	4	0.6	2	7.6	4.8	4.8	1/5
V	4	0.6	2	7.6	5.4	5.6	1/5
JE	4	0.6	2	7.3	5.6	5.6	1/5
JCC	4	0.6	2	7.3	6.1	6.1	1/5
JE	4	0.6	5	7.3	5.5	5.7	1/5
JCC	4	0.6	5	7.3	6.1	6.0	1/5

not the only factor influencing the production of toxin by the two strains JE and JCC. In these two examples the *pH* did not change materially when the serum content of the medium was raised from 2 to 5 per cent, but the toxicity of the product was doubled in the one case and quadrupled in the other. By adjusting the initial *pH* and the sugar content of the medium, strains could be induced to grow with the evolution of a particular *pH* cycle, but the conditions evoking a given cycle varied with the different strains. The addition of a higher concentration of serum increased the toxicity, as judged by the M L D, in certain but not in all strains.

Working from this basis it was possible to group the strains in

regard to the intravenous M.L.D. of their toxin for mice and to the growth conditions in which it had appeared.

Two groups could be differentiated, based on the capacity to produce acid from fermentable sugar. Group 1, producing less acid under these conditions, contained three very toxic strains—the well-known S107 and two newly isolated strains JCC and JE—all showing an M.L.D. of $\frac{1}{40}$ – $\frac{1}{80}$ c.c. With JCC, the most typical member of the group, the sugar content could be increased to 0.8 per cent., which was beyond the optimum for toxin production, without the development of an acidity greater than pH 6.0 at 4 hours and 6.2 at 7 hours. JE was less distinctive in this respect but belonged to the group nonetheless and required a high sugar content to produce an acidity equal to pH 5.6. Group 1 strains all needed a high serum content or added meat extract to give the best results.

Group 2 contained the great majority of the strains tested and, while here too the acidity produced varied a good deal with the strain, pH values of 5.4 and 4.8 were registered with sugar contents of 0.45 and 0.6 per cent.

The majority of the strains produced their best toxins at a final pH (after 6–7 hours' growth) of 6.0 to 6.2, even though a certain proportion could also yield good toxin at more acid values (table II).

TABLE II

Correlation of pH values developed during growth with maximum toxin yields in 45 cultures from 25 strains

Period of growth	pH value associated with maximum toxin yield	No. of examples	Percentage
3–4 hours	5.5–5.6	2	4.4
	5.7–5.9	12	26.7
	6.0–6.2	28	62.2
	6.3–6.4	3	6.6
6–7 "	5.7–5.8	5	11.1
	6.0–6.2	30	66.7
	6.3–6.5	10	22.2

Those strains which could produce their toxin through a relatively wide pH range were on the whole the more regularly toxic, while the more variably toxic strains occurred mostly among those whose toxin-producing capacity seemed to be more affected by the pH cycle.

Table III gives examples of the relation of toxin production to the acidity registered in the medium by three of the more toxic strains of the type described above. This question of the regular production of toxin seemed to be of some importance in relation to

all these factors and also to the clinical histories, as will be seen below

TABLE III

Conditions in which toxin was produced

Strains and origin	M L D at 6 hours (c c)	pH at	
		4 hours	6 hours
SR 12			
Fatal fulminating gas gangrene in wound 1915	$\frac{1}{10}$ $\frac{1}{10}$ } $\frac{1}{25}$	5.6 6.1 5.4	5.7 6.3 5.4
V			
Fatal gas gangrene air raid casualty, 1940	$\frac{1}{10}$ $\frac{1}{10}$ } $\frac{1}{10}$	6.0 6.2 5.9 5.4	6.2 6.2 6.2 5.6
DM			
Wound infection without gas gangrene 1940	$\frac{1}{10}$ $\frac{1}{10}$	6.1 4.8	6.2 4.8

Of the 26 strains tested, 17 were regularly toxic in the sense that they yielded toxin within the range of potency characteristic for the strain whenever tested in suitable conditions, and 8 showed a more irregular capacity to produce toxin. In the absence of more exhaustive tests it was not quite clear whether this irregularity was really characteristic of the strain or due to failure to find the optimum conditions for growth. It can, however, be concluded that they are less robust in their capacity to produce toxin and that their needs are more difficult to meet.

The stability of the strains in regard to toxin production over a longer period of cultivation *in vitro* has not been gone into here. The new strains were mostly tested within a few weeks of isolation. *Cl. welchii* is notoriously liable to throw off variants, as has been shown by McGaughey (1933), Prigge (1937) and Henderson (1940). On the other hand certain strains, such as SR 12—isolated in 1915—and S107, are extremely stable in regard to the maintenance of their toxicity.

The production of toxin in this kind of culture seemed to depend upon rapid initial growth reaching a suitable pH value at the peak of fermentation and a maintenance of growth for some adequate period after this, with a stabilisation of the pH value or a slight movement only towards the more alkaline side. If the medium will not promote the initial rapid growth with accompanying increase of acidity within the optimum range the amount of toxin is reduced. The medium must be rich enough in nutrient material to support growth during 2-3 hours after the peak of fermentation.

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In the clinical group I (15 cases), where gas gangrene was present, were found the five most toxic strains (40-80 M.L.D. per c.c.). The two most toxic strains (JCC and JE), which produced 80 M.L.D. per c.c., came from severe cases of gas gangrene arising in the leg; these cases were successfully treated with final recovery.

TABLE IV

Comparison of toxicity within the three clinical groups

Clinical group	Strains in descending order of toxicity	M L D (c c)	Source	Outcome of case
I Gas gangrene	JCC	$\frac{1}{80}$	Secondary arm abscess after leg amputation	Recovery
	JE	$\frac{1}{80}$	Gas gangrene of thigh	"
	SR 12	$\frac{1}{40}$	Fulminating gas gangrene (1915)	Death
	V	$\frac{1}{40}$	Massive gas gangrene; multiple leg wounds	Rapid death
	CB (pleura)	$\frac{1}{40}$	Severe gas gangrene of abdomen and chest	Recovery
	CB (liver)	$\frac{1}{40}$	Severe gas gangrene of abdomen and chest	"
	A 72	$\frac{1}{40}$	Gas gangrene of leg under plaster, no fracture	Death
	R	$\frac{1}{40}$	Massive gas gangrene	"
	CL	$\frac{1}{40}$	Gas gangrene	Recovery
	LP	$\frac{1}{40}$	" " of thigh	"
	M	$\frac{1}{40}$	" " " thigh	"
	BP	$\frac{1}{40}$	" " " leg	"
	ST	$\frac{1}{40}$	" " " thigh and leg	"
	E	$\frac{1}{40}$	" " "	"
	A 8	$\frac{1}{8}$	Mixed peritonitis with gas gangrene	Death
II. Infection without symptoms	DM	$\frac{1}{40}$	<i>Cl. welchii</i> infection; war wound, no details	Recovery
	CT	$\frac{1}{40}$	<i>Cl. welchii</i> infection of compound fracture	"
	BL	$\frac{1}{40}$	<i>Cl. welchii</i> infection of lumbar wound	"
	G	$\frac{1}{40}$	<i>Cl. welchii</i> infection, war wound, no details	"
	LY	$\frac{1}{40}$	<i>Cl. welchii</i> and mixed pyogenic infection of buttock	"
III. <i>Cl. welchii</i> present without establishment	A 71	$\frac{1}{40}$	Scalp wound	"
	W	$\frac{1}{40}$	Wood chip in orbit	"
	H	$\frac{1}{40}$	Pleural clot around shrapnel	"
	C	$\frac{1}{40}$	Chest wound	"
	K	$\frac{1}{40}$	Amputation stump	"
	D	$\frac{1}{40}$	Plaster encased wound (after 6 weeks)	"

Of the three other strains in this most toxic group, two were from rapidly fatal cases of gas gangrene and produced toxins with 50 and 40 mouse M.L.D. per c.c. respectively, and one (CBP) from severe gas gangrene of abdomen and chest with excellent recovery (40 mouse M.L.D. per c.c.).

In the clinical group I there was a sub-group of eight cases where the associated strains of *Cl. welchii* showed a toxicity equal to 10-20 mouse M.L.D. per c.c. This is a very frequent level of toxicity for these *Cl. welchii* strains. Among the cases of clinical gas gangrene arising from strains at this level of toxicity were two fatal cases, R, a shunting injury which developed rapidly fatal massive gas gangrene, and A 72, which was treated in plaster and died in 48 hours. Both these cases yielded strains with a toxicity of 20 mouse M.L.D. per c.c. and are of importance as showing that organisms of this quite ordinary degree of toxicity can produce rapidly fatal infections of the massive type where conditions are favourable to their development.

Group II in table IV, where there was evidence of proliferation of the organisms without symptoms of gas gangrene, contained five strains which range from the highly toxic DM producing 40 M.L.D. per c.c. to G whose toxin contained 5 M.L.D. per c.c.; in addition, the only practically non-toxic strain LY came into this clinical group. The interest here lies in the fact that, in spite of the possibility for some degree of proliferation being present, the organisms did not find the necessary conditions of anaerobiosis, food supply, etc., to create the massive growth with evolution of toxin which alone seems to enable them to attack living tissue and set up gas gangrene. This failure could not be attributed to the basic absence of toxicity of the strain DM, which was regularly toxic, producing at its best 40 M.L.D. per c.c.

The third clinical group (III), where *Cl. welchii* was fortuitously present in the wound, contained six strains yielding toxins of 10-20 mouse M.L.D. per c.c.; that is to say they were in the big group of strains of average toxicity. These six strains were in conditions affording no opportunity of setting up a true infection and constitute really only a small random sample of the kind of *Cl. welchii* found among wounded persons. Strain D is, however, of rather particular interest, as it was obtained from under a plaster dressing after 6 weeks. It was surviving in conditions which did not permit of its development. This seems to be an example of *Cl. welchii* unable to take hold, not in spite of nor because of the plaster, but because of the state of the wound.

It seems justifiable to conclude that, in general, the most regularly toxic strains capable of secreting toxin through a fairly wide pH range were also the most effective in causing gas gangrene.

On the evidence supplied by table IV all the strains tested, except only LY, seemed to be of a basic toxicity sufficient to cause gas gangrene if the state of the wound was such as to permit of rapid proliferation and evolution of toxin. *Cl. welchii*, it should be repeated, has never shown itself capable of an attack on sound tissue, which is the essence of gas gangrene, unless it is spreading

out from a focus in which it meets conditions—reduced oxygen tension and an abundant food supply—permitting rapid growth with the evolution of toxin. Telling examples illustrating this are afforded by the three cases A72, DM and D. A72 was a rapidly fatal case of gas gangrene arising from multiple puncture wounds, which was encased in plaster within a few hours of wounding. The organism, with a toxicity of 20 mouse M.L.D. per c.c., had all it needed to multiply vigorously and produce a rapidly advancing gas gangrene undisturbed by any interference. DM, a very regularly and highly toxic strain (40 M.L.D. per c.c.), proliferated harmlessly, lacking the conditions for secreting its toxin, and in spite of its basic capacity in this direction was quite ineffective. The strain D already mentioned adds the third example and, although lying undisturbed under plaster, it did not have the conditions for good growth and was quite innocuous.

This method of investigation is not a very rapid or economical way of appraising the toxicity of strains, as it requires a number of tests to explore the nature of the strain and find the best level of toxin production. Moreover, the number of mice required is very considerable. It does, however, reveal the nature of the strain and, if it is carefully used, a good idea of the toxicity and general capacity of the organisms can be obtained. An application of the lecithovitellin test would no doubt provide a more economical method when the reaction has been further explored and a reliable equation of the flocculation and opalescence to lethal action has been established (Macfarlane *et al.*, 1941).

SUMMARY

Twenty-six strains of *Cl. welchii* recently isolated from air raid casualties and wounded soldiers were tested for their capacity to produce toxin *in vitro*. The method of toxin production is described.

The yield varied from 5 to 80 intravenous mouse M.L.D. per c.c., the majority (61.5 per cent.) of strains tested showing a toxicity of 10-20 mouse M.L.D. per c.c.

The strains were isolated from cases which ranged from acute gas gangrene to those in which the organism had only been transiently present without creating any recognisable symptoms in the patient. All but one of the strains tested were of a basic toxicity equal to and above that shown to be capable of causing gas gangrene if the conditions in the wound were favourable to its development.

Apart from the valuable co-operation of the pathologists of the Emergency Medical Service already referred to, the authors have received a great deal of assistance from other workers. We are particularly indebted to Professor T. Dalling and Mr C. A. McGaughey of the Institute of Animal Pathology, Cambridge, for fundamental advice on the whole problem of toxin produc-

tion. Very valuable help was also received from Dr B. C. J. G. Knight, Mr R. S. Roberts and Dr D. Riding in the matter of suitable media. We have pleasure in expressing our thanks to Dr W. E. van Heyningen of the Biochemical Laboratory, Cambridge, for constant helpfulness and co-operation in many directions.

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INTIMAL CORONARY ARTERY HÆMORRHAGE AS A FACTOR IN THE CAUSATION OF CORONARY OCCLUSION

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(PLATES IV AND V)

MANY theories have been propounded to explain the exact mechanism of coronary occlusion. Although these have varied somewhat, all are agreed that in the vast majority of cases the occlusion occurs in vessels which are the seat of some degree of atherosclerosis.

Koch and Kong (1932-33) suggested that stasis and eddying of blood occurring near atherosclerotic plaques are sufficient to precipitate thrombus formation even when the endothelial lining is intact. This stasis is enhanced by the reduced blood flow of congestive heart failure, whether brought about by the coronary atherosclerosis or by decompensation in an associated hypertension.

In recent years greater attention has been paid to changes in the vessel wall at the site of occlusion in an endeavour to ascertain the exact mechanism of occlusion or to discover what changes exist which might initiate intravascular thrombosis. Thus Boyd (1928) described an acute inflammatory reaction in the intima of two cases of recent occlusion and suggested this as a possible factor in all cases. Leary (1934-1938), who noted breaks in the integrity of the overlying tissue in atheromatous plaques, suggested that the rupture of so called atheromatous abscesses was a common pre thrombotic lesion, especially in old people. A curious fibrinoid lesion has also been noted at the site of occlusion by many workers, including Leary (1936) and Clark *et al* (1936). It has been variously interpreted as tissue necrosis or a fibrin coagulum or clot.

Paterson (1936) and Wartman (1938) have suggested that hæmorrhage into the intima from intimal sinusoidal vessels might be an important precipitating factor in intravascular coronary thrombosis. Their work is supported by the fact that a similar process seems to occur in atherosclerotic cerebral vessels—where it leads to thrombosis—and also in pulmonary vessels. Hæmorrhages into the intima had, of course, been noted before but were differently interpreted. Boyd considered that the presence of the blood was merely part of the exudative inflammatory reaction, while Leary thought it was the result of regurgitation from the lumen into the intima following rupture of an atheromatous abscess.

In an endeavour to discover the exact mechanism of coronary artery occlusion, seventeen recent cases have been studied.

Material and methods

The material was derived from autopsies carried out in this department over a period of about two years. The heart in every case of suspected coronary occlusion was examined systematically. After the external surface had been scrutinised and described, the coronary arteries were cannulated and the coronary system filled with radio-opaque Röntgen solution at a pressure of 100-200 mm. of mercury. The arteries were then tied off and the site of occlusion located by x-ray examination. The affected vessel was carefully dissected off and blocks were taken from the occluded portion. These, after fixation in formol-Zenker, were embedded in paraffin in the usual manner. A few of the blocks required preliminary decalcification. Sections were stained with hæmatoxylin and eosin, hæmatoxylin and van Gieson, Masson's trichrome stain and Verhoeff's elastin stain. Sections were also treated to show the prussian blue reaction.

Description of the coronary artery lesion in eleven cases of occlusion associated with intimal hæmorrhage

Case 1. The *adventitia* shows diffuse fibrous thickening, while the *media* is much thinned and fibrosed. There are well marked *intimal* atherosclerotic changes and in the deeper aspects there are large sinusoidal blood spaces surrounded in many instances by chronic inflammatory changes. The lumen of the vessel is considerably reduced by a large eccentric plaque of atheroma. Recent hæmorrhage is evident in the soft detritus of the plaque and the overlying vascular endothelium has been raised, reducing the lumen to a mere slit. There is no evidence of superimposed thrombosis.

Case 2. There is well marked concentric fibrosis of the *adventitia*, with infiltration by lymphocytes and plasma cells. This infiltration shows a tendency to collect around some of the blood vessels but there is no evidence of endarterial or periarterial changes in the *vasa vasorum*. The *media* is thinned, fibrosed and infiltrated by lymphocytes. The *intima* is thickened and shows in its deeper layers a large atheromatous plaque with partial calcification. Many large sinusoidal blood vessels surrounded by chronic inflammatory cells and much fibroblastic activity are present in the *intima*. Evidence of former perivascular hæmorrhages is shown by the presence of hemosiderin-containing macrophages in relation to some of the vessels. A recent hæmorrhage is present in the immediately subendothelial zone and overlying it is a dense fibrin clot from which the final thrombosis appears to have arisen. This hæmorrhage has extended slightly into a nearby atheromatous plaque which contains red cells and fibrin. The overlying fibrous tissue and endothelium have been broken through and the lumen is apparently in direct communication with this area of atheroma.

Case 3. The *adventitia* shows slight fibrous thickening and there is, over one segment of the vessel, a marked adventitial infiltration of chronic inflammatory cells, including many plasma cells. The *media* is thin and markedly fibrosed, while over some segments it is practically non-existent. The *intima* is thickened and fibrosed and shows the presence of an atheromatous plaque containing many rounded fat-laden macrophages. In the deeper layers many sinusoidal blood vessels are present. In the *intima* deep to the adventitial infiltration above-mentioned there is a lymphocytic perivascular infiltration with some fibroblastic activity. In the atheromatous plaque there is a recent hæmorrhage and the overlying *intima* has been

raised and apparently broken at an angle without showing any extension of the hæmorrhage into the lumen. Secondary thrombosis is present in the lumen, but has not completely occluded it in the sections examined.

Case 4 The *adventitia* is grossly thickened and equals in width the sum of the other two coats. This is entirely due to the presence of dense collagenous fibrous tissue, it contains very few cells. The *vasa vasorum* in the *adventitia* show typical syphilitic endarterial and periarterial changes. The *media* has been almost completely replaced by active fibrous tissue, muscle being present in only one segment of the vessel wall. The *intima* is thickened and fibrosed. It shows many new blood vessels in its deeper layers, surrounded by a cellular infiltration which includes many plasma cells. Also in this perivascular zone there is active fibroblastic proliferation and evidence of old perivascular hæmorrhages. The fibrous tissue in the immediately subendothelial zone is still very active and of granulation tissue type. In this zone there is a recent hæmorrhage and over it a dense fibrin clot, while the rest of the lumen is occluded by looser thrombus.

Case 5 The branches of the *vasa vasorum* lying in the *adventitia* have good muscular walls and show no pathological changes of note. Focal collections of chronic inflammatory cells—lymphocytes and some plasma cells—infiltrate the fibrous tissue of the *adventitia* but do not appear to bear any significant relationship to pathological processes occurring in either *media* or *intima*. Hæmosiderin deposits are present in relation to some of the smaller *vasa*. The *media* is much thinned and fibrosed and in some segments is reduced to a few strands of muscle. In the *intima* there are marked atheromatous deposits which encircle the lumen and fibrosis of the overlying subendothelial tissue. In the deeper layers of the *intima*, vascular spaces can be readily identified and many new capillaries are seen. A chronic inflammatory change is present in this zone, with cellular infiltration, fibroblastic proliferation and the formation of new capillaries, while hæmosiderin deposits give evidence of old hæmorrhage. A more recent hæmorrhage has occurred into the atheromatous plaque and has spread completely round the lumen. This has raised the intimal covering of the plaque and reduced the size of the lumen, while recent thrombus has completed the process of occlusion.

Case 6 The *adventitia* is thickened over some segments and shows focal collections of chronic inflammatory cells predominantly lymphocytes. Hæmosiderin laden macrophages are also present. The *intima* is abnormally thick, due to the presence of abundant dense collagenous fibrous tissue, while atheromatous cholesterol clefts are almost non-existent. In the deeper aspects of the *intima* many sinusoidal blood vessels are present which are surrounded by a zone of active fibroblastic proliferation and chronic inflammatory cell infiltration. In some of these areas hæmosiderin laden macrophages are numerous around the vessels. Many of these intimal changes occur deep to the focal adventitial infiltrations and direct continuity can sometimes be traced through the *media*. In the more superficial aspects of the intimal fibrous layer there is a recent hæmorrhage, closely related to some sinusoidal blood vessels. It has torn up the fibrous tissue in the neighbourhood and has raised up the immediately subendothelial fibrous tissue from its bed. The latter shows well marked fibrinoid change. A recent thrombus completely occludes the lumen and the oldest part of this clot is attached to the wall at the site of the intimal hæmorrhage.

Case 7 The *adventitia* shows no significant histopathological changes. The *media* is everywhere intact and shows only a mild degree of fibrosis. The *intima* is the seat of advanced atheromatous change, with accompanying fibrosis. Calcification is present and there are many sinusoidal blood spaces.

in the deeper layers. A recent subendothelial hæmorrhage almost completely encircles the vessel. It has raised up the overlying fibrous tissue and may have broken through into the lumen at one point. A recent thrombus completely occludes the lumen and is attached to the wall at a point which overlies an area of marked intimal hæmorrhage.

Case 8. There are many focal collections of chronic inflammatory cells throughout the *adventitia*. In one section there is marked fibroblastic activity over one segment of the *adventitia*, with extension through and destruction of the *media*. The *media* is thinned out and much fibrosed. The *intima* is greatly thickened and fibrosed and shows subendothelial deposits of fat which has been largely taken up by globular macrophages. In one of the sections showing fibroblastic adventitial activity and medial destruction there is a small area of necrosis in the deeper layers of the *intima*, with many pyknotic nuclei, resembling an intramural abscess. It is surrounded by a zone of active fibroblastic proliferation. A recent hæmorrhage is present immediately beneath the endothelium and extends almost half-way round the circumference. It has ruptured secondarily into the lumen, which is completely occluded by recent thrombus.

Case 9. The *adventitia* shows considerable fibrous thickening unevenly distributed around the vessel. In this fibrous tissue focal collections of chronic inflammatory cells are prominent, including many plasma cells. The *media* shows thinning and fibrosis. The *intima* is the seat of well marked atheromatous changes, with attendant fibrosis. Many large sinusoidal blood vessels are present in its deeper layers. Nearly all these vessels are surrounded by an inflammatory reaction which consists of lymphocytes, plasma cells and some polymorphs, while evidence of active fibroblastic proliferation is marked. In some cases perivascular collections of hæmosiderin-containing macrophages are present. A dense whorled deposit of fibrin is present in the wall near the lumen and in close association with a group of sinusoidal vessels showing marked perivascular inflammatory changes. This fibrin shows commencing organisation at its periphery but is in direct communication with the lumen, which is occupied by recent thrombus.

Case 10. Focal collections of chronic inflammatory cells are present in the *adventitia*, including many plasma cells. Fibroblastic activity is evident nearby and perivascular macrophages containing hæmosiderin are evident. The *media* is thinned, fibrosed and in some areas infiltrated from the adventitial cellular collections. The *intima* is the seat of most advanced atheromatous change, reducing the diameter of the lumen considerably. In the deeper layers, which still remain fibrous, there are many sinusoidal blood spaces surrounded by a zone of fibroblastic overgrowth and inflammatory cell infiltration, including some polymorphs and plasma cells. Evidence of previous hæmorrhage from such vessels is shown by hæmosiderin deposits. Throughout the ground substance of the atheromatous plaque—which appeared brown to the naked eye—there is a curious fibrinoid staining reaction. Immediately beneath the vascular endothelium but separated from it by a few collagenous strands, there is a recent hæmorrhage which almost surrounds the lumen and has ruptured through its overlying fibrous covering to enter the lumen. The latter is itself completely occluded by recent thrombus.

Case 11. The *adventitia* contains focal collections of chronic inflammatory cells, including many plasma cells. The *media* is much thinned and fibrosed. The *intima* shows advanced atheromatous deposits. In its deeper layer close to the *media*, where fibrosis is marked, there are many sinusoidal blood vessels surrounded by chronic inflammatory changes. A recent hæmorrhage is present in the *intima* and has extended into the lumen, which is occluded by recent thrombus.

Commentary

Of the eleven vessels in which occlusion was due, in part at least, to intramural hæmorrhage, ten showed the presence of advanced coronary atherosclerosis. In many, this was associated with intimal calcification. In one case only, the occluded vessel was the seat of syphilitic arteritis which had extended from a nearby syphilitic aortitis into the ostium and first cm. of the right coronary artery. This case of acute coronary occlusion occurred in a woman of 34 who died suddenly while at work and about whom no clinical details are available. The right coronary artery was completely occluded at its mouth by a recent intramural hæmorrhage superimposed on the syphilitic changes. It is significant that the same process, leading to final occlusion, should have occurred both in vessels affected by syphilis and in those the seat of atherosclerosis.

Syphilis of the coronary arteries usually consists of involvement of the coronary ostium, especially the right. The involvement rarely extends far down the vessel and it is common to have syphilitic involvement of its mouth with atherosclerotic changes elsewhere.

The process of syphilitic coronary artery closure has always been a problem about which there have been many theories but few facts. It is usually believed that the endarteritic change proceeds slowly and inexorably to final occlusion, and we have recently seen a case of syphilitic aortitis and aortic valvulitis where the free edges of the valves were glued back to the aortic wall and the right coronary ostium was completely occluded without any macroscopic changes in the myocardium supplied by this vessel. In the single instance in this series, however, an acute occlusion had occurred at the ostium of the right coronary artery, which was the seat of syphilitic change. This allowed us to see the actual process of acute coronary artery closure in such circumstances. The vessel, which showed marked adventitial and intimal thickening, had an obvious macroscopic intramural hæmorrhage and the lumen was occluded by thrombus. Histologically there was widespread syphilitic arteritis, the adventitia being greatly thickened and showing specific vascular and perivascular changes. The media was thin and in many places destroyed, while the intima was greatly thickened, due to the formation of soft granulation tissue in which atheromatous changes were entirely absent. The whole wall including the intima was exceedingly vascular. There was clear evidence of previous hæmorrhage from the intimal capillaries in the shape of perivascular collections of hæmosiderin-containing macrophages, while a recent gross hæmorrhage had occurred into the intima. A thrombus which only partially occluded the lumen at its mouth but which, more distally, did so completely, was attached to the intima over the area of intramural hæmorrhage. It would appear therefore that

intramural hæmorrhage is not invariably due to atherosclerosis: it may result from inflammatory as well as degenerative change. The underlying disease, moreover, is, in many cases at least, merely predisposing and not precipitating.

There is little agreement in the literature on the problem of the vascularisation of the coronary artery wall, and the main conflict has centred around the blood supply of the intima. The general consensus of opinion appears to be that the normal intima of the human coronary is not vascularised (Paterson, 1936; Leary, 1938) but is supplied with blood by imbibition from the lumen of the main vessel, while the deeper aspects are supplied by a few vessels penetrating through from the media. Winternitz *et al.* (1938), however, have demonstrated a vascular network in the normal intima of various animal species. By means of a special injection technique they were able to demonstrate vascular spaces in the vessel wall derived from three distinct sources—the adventitia, the region of the orifices of branches and the lumen of the vessel itself. They considered that such spaces must exist also in the normal intima of man, although they were unable to demonstrate them by their technique. These vascular spaces, few in youth, increase with age and are very marked in arterial diseases such as atherosclerosis.

On the other hand, there is complete unanimity as to the fact that atherosclerotic vessels are well vascularised and that this vascularity includes the intima. The blood vessels found in the intima of atherosclerotic vessels are derived from two sources. Those on the deeper aspect of the intima, in close proximity to the media, are derived from the vasa vasorum of the adventitia. This fact, demonstrated by Winternitz *et al.* and many others, was amply confirmed during the course of the present investigation. The more superficial vessels have been traced by serial section and by injection to the lumen of the coronary artery (Leary, 1934; Paterson, 1936; Winternitz *et al.*, 1938). The development of an intramural vascular circulation is apparently dependent on inflammatory vascular changes or atherosclerotic degenerative changes.

Histological examination of the coronary vessels showed that a capillary circulation was universal in the intima of those arteries which were the seat of pathological processes, whether inflammatory or degenerative. These vessels were situated to a large extent in the deeper part of the intima in close apposition to the media, but were also present immediately beneath the endothelium. It is difficult to visualise the occurrence of vessels within an area of atheroma: such lesions are probably, as Leary (1934) suggests, purely avascular, although the presence of vessels in close association with them is constant. It was usually found that the areas with least atheroma and most fibrosis showed the greatest vascularity, while areas with advanced atheromatous lesions and wide cholesterol

clefts were relatively avascular. Blood vessels were always found, however, in the margins of these advanced lesions.

Since the normal intima is not vascularised while the pathological intima is, it would seem that vascularisation is a response to rather than a cause of inflammatory or atherosclerotic change. It is capable, nevertheless, of maintaining and increasing the intimal fibrosis and, since it leads to intramural hæmorrhage, plays an important role in the causation of coronary occlusion.

The association of adventitial lymphocytic infiltration with intimal atheromatous deposits is frequent. Such infiltration tends to be perivascular and to consist in part of plasma cells, and as it is often associated with many newly formed vasa vasorum, the question of syphilis may be raised. Syphilis, however, rarely extends beyond the coronary ostia and is associated with a syphilitic aortitis, while the vasa vasorum show obliterative endarteritis in addition to the perivascular changes.

In most of the vessels of this series it was noted that the intimal sinusoidal vessels, especially the more deeply placed, showed well marked perivascular changes, consisting of tissue necrosis, lymphocytic infiltration and, in some instances, fibroblastic activity. Around some of the vessels previous hæmorrhage was indicated by the presence of collections of hæmosiderin-containing macrophages. If these had been found around all the vessels showing the perivascular changes above referred to, one would have had no hesitation in saying that the changes in question had resulted from organisation of previous hæmorrhages. The fact that similar changes could occur without hæmorrhage suggests on the other hand a common ætiological agent. Histological evidence points to the process being inflammatory and suggests that it may either end in healing with fibrosis or proceed to intramural hæmorrhage by toxic damage to the capillary wall.

This raises once more the question of the relationship of infection to the development of sclerosis of arteries, which, entirely discounted by MacCallum (1925), has again been mooted by Winternitz *et al.* These authors, by demonstrating a vascular bed in blood vessels, have altered our point of view as regards the reaction of vessels to injurious agents, bringing the pathological changes occurring in vessels into line with similar changes in other tissues. Thus instead of regarding certain of the pathological processes which occur in vessels as wholly degenerative in nature, they could perhaps be better understood as some variant of the ordinary exudative and reparative processes which occur elsewhere in the body in response to injury. The flaw—if such it be—in the argument put forward by these authors is in deducing from changes observed late in the disease that these changes are important as ætiological factors in the development of the disease process. There is as yet insufficient

evidence to show that exudative changes ever occur early in the development of atherosclerosis, although they commonly accompany the fully developed disease and are probably of importance in increasing the vascular fibrosis and, by the formation of new fragile capillaries, in enhancing the liability to intramural hæmorrhage.

Hæmorrhage from intimal capillaries into the wall of an atherosclerotic coronary artery is a common occurrence and in many instances is unassociated with any thrombus formation in the lumen (fig. 3). When fresh, the relationship of such hæmorrhages to intimal vascular channels can be readily distinguished; later the extravasated blood becomes organised, numerous new capillaries appear, fibroblastic activity and lymphocytic infiltration become evident and the blood pigment is taken up by macrophages in the form of hæmosiderin.

Various interpretations have been placed on the presence of extravasated blood in the wall of the coronary arteries and most authors consider that it is derived from blood in the lumen as a result of breaks in the integrity of the endothelium (Benson, 1926; Koch and Kong, 1932-33). Boyd (1928) thought that the blood was part of an inflammatory exudate in the wall, while Leary (1936) described the rupture of atheromatous abscesses into the lumen and the subsequent entrance of blood into the interior of the lesion.

Paterson (1936) first suggested that an intramural hæmorrhage of this kind was the primary process in coronary obstruction and that it might subsequently lead to intraluminal thrombosis (figs. 1 and 2). This has been confirmed by Wartman (1938), Winternitz *et al.* (1938) and Horn and Finkelstein (1940).

In this small series of 17 cases there was evidence that recent intramural hæmorrhage was directly or indirectly responsible for the coronary occlusion in 11, and in 6 of these there were relics of previous hæmorrhage in relation to blood vessels situated deep in the intima (figs. 4 and 5). These deeper hæmorrhages, because of their position, would be unlikely to cause changes which might lead to immediate coronary occlusion, and the survival of the patient would allow time for healing processes to occur, with the probable formation of further new capillaries in the granulation tissue. As such newly formed capillaries have been shown to be still less resistant to intravascular pressure, it is apparent that they will tend to rupture more readily and so set up a vicious circle which goes on until the vessel becomes occluded, either by the formation of a large intramural hæmatoma or by a superimposed coronary thrombosis. It is therefore suggested that during the course of his life a patient with coronary atherosclerosis will be subject to these recurring intimal hæmorrhages but that usually it is only when the hæmorrhage is either massive or superficial, thus causing secondary damage to the

CORONARY OCCLUSION

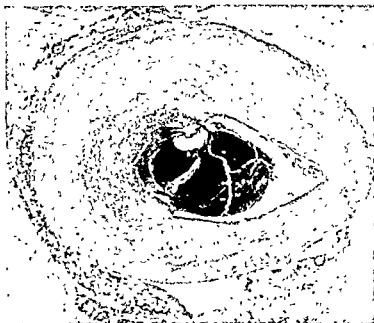


FIG. 1.—Coronary artery showing advanced atheroma with intimal hemorrhage and attached overlying thrombus. H. and E. $\times 25$.

FIG. 2.—Coronary artery showing advanced atheroma and superficial intimal hemorrhage which is continuous with thrombus in the lumen. Note large sinusoidal capillaries in the thickened intima. H. and E. $\times 25$.

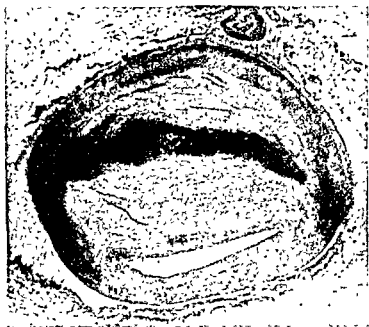


FIG. 3.—Coronary artery showing advanced atheroma and hemorrhage deep in the intima. This has resulted in almost complete occlusion without the formation of an intraluminal thrombus. H. and E. $\times 25$.

CORONARY OCCLUSION



FIG. 4.—Part of the wall of a coronary artery showing recent hæmorrhage, hæmosiderin containing macrophages and an inflammatory infiltration H. and E. $\times 75$

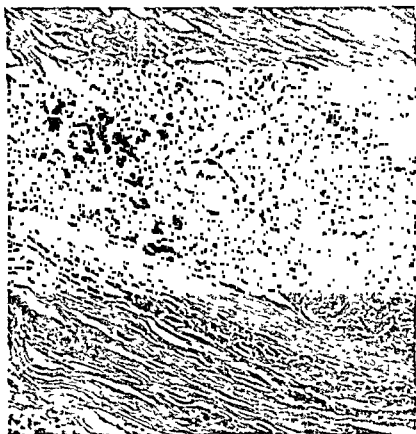


FIG. 5.—Section showing an area in the deeper part of an atheromatous plaque. Numerous hæmosiderin containing and inflammatory cells are present and several small blood vessels can be seen. H. and E. $\times 80$

action, whether local or general. In the present material, histological examination afforded ample evidence of chronic perivascular inflammatory change suggestive of the action of blood-borne toxins. There is no available evidence, except perhaps in the case of syphilis, to indicate the nature of these hypothetical toxins.

Lastly the integrity of the capillary is dependent on the rigidity or otherwise of the supporting stroma. This has been stressed by Paterson (1936) and others as the most important factor in the production of intimal hæmorrhages. The capillaries are usually found to lie close to or in an area of atheromatous degeneration and are thus ill-supported by the surrounding tissue. The capillaries become dilated by the intravascular pressure and finally, because of increasing capillary fragility due to age or toxic action, rupture occurs and the blood pours out into the surrounding area of atheroma. In the material examined, however, the intimal sinusoidal vessels rarely showed any lack of support by the surrounding stroma, which in most cases consisted of dense collagenous bands.

It is therefore considered that the most important factors concerned in the production of intramural coronary artery hæmorrhages are weakening of the capillary wall and sudden raising of the intracapillary pressure. As regards the latter, evidence derived from the clinical examination of a group of cases points to exertion and emotion as the main precipitating factors. These clinical conclusions cannot be considered anything more than suggestive, because of the possible painless nature of the original intramural hæmorrhage and the progressive *tempo* of the changes leading to the final block. That intramural hæmorrhage can occur in both atherosclerotic and syphilitic coronary disease without causing occlusion indicates that some additional factor may be necessary to bring about the final catastrophe.

Wartman (1938) has shown that in many cases, if the hæmorrhage is extensive, it is itself capable of causing coronary occlusion. In these cases the bleeding usually occurs into the substance of a fatty atheromatous plaque, causing a large intramural hæmatoma which occludes the lumen. This fact was confirmed in two of the cases examined, where an extensive intramural hæmorrhage had involved a nearby plaque, and by raising the overlying endothelium and subendothelial connective tissue had occluded the lumen without any superimposed thrombosis.

In a search for secondary factors Clark *et al.* (1936) and Paterson (1936) found that, if the hæmorrhage occurred into the wall proximal to a stenotic plaque, intravascular thrombosis and occlusion were common. Here the stasis and eddying of blood which occur in this situation will favour intravascular thrombosis if the necessary precipitating factor in the shape of damaged endothelium is added.

The intramural hæmorrhage is itself capable of producing this endothelial damage.

Paterson (1938) has described a number of ways in which the thrombosis may be initiated—diffusion of blood and thromboplastic substances from a superficial hæmorrhage, necrosis of the overlying intima and endothelium, or retrograde thrombosis of capillaries derived from the lumen.

In this series nine out of the eleven cases with intimal hæmorrhage showed superimposed thrombosis, and in many of them the oldest part of the thrombus could be identified attached to the wall overlying a subendothelial hæmorrhage. In others the blood from an intramural hæmorrhage could be seen to have ruptured through the covering endothelium into the lumen of the artery, where it became continuous with a thrombus in the main channel. In one case, in addition to a recent intimal hæmorrhage, there was an organising clot of some age, lying in the wall but directly continuous with a thrombus in the lumen. This would tend to show that the process of occlusion is not in all cases sudden and acute but may be slowly progressive.

The fibrous tissue in relation to an intimal hæmorrhage, especially the tissue lying between the hæmorrhage and the lumen, is often the seat of fibrinoid change. This is taken to be a degenerative phenomenon.

Many cases of acute coronary occlusion follow immediately some degree of exertion and the pathological basis for these cases is probably the formation of a large intramural hæmatoma which has caused complete occlusion. Many others are apparently unrelated to exertion or emotion and occur during rest. It is possible that in these cases, during a phase of raised blood pressure induced by exertion, a small intramural hæmorrhage occurred, insufficient in itself to occlude the vessel, but producing degenerative changes in the overlying endothelium and initiating intravascular thrombosis. Such a thrombus is more likely to form during a period of rest. Thus, although the intimal hæmorrhage may occur during a phase of raised blood pressure induced by emotion or exertion, it may take hours or even days for the complete occlusion to develop and the final intravascular thrombosis may occur during a period of bodily rest.

Summary

1. Changes in the vessel wall are considered to be of greater importance in the development of coronary occlusion than changes in the blood.
2. The most common predisposing disease is coronary atherosclerosis.
3. Many sinusoidal blood vessels are found in relation to intimal

atheromatous plaques. These vessels occur in two situations, either deep in the intimal tissues close to the media, or more superficially, near the endothelium. In the majority of cases they are surrounded by chronic inflammatory changes.

4. Hæmorrhage from these sinusoids is a not uncommon finding in coronary atherosclerosis. Hæmorrhages into the deeper zones of the intima heal by granulation tissue in which new capillaries are numerous. Such a process increases the fibrosis and the vascularity of the intima and predisposes it to further hæmorrhage.

5. Superficial intimal hæmorrhage is a most important factor in the ætiology of coronary occlusion. It was present in 11 out of 17 cases examined, but in 9 of these, thrombotic occlusion of the lumen was also present.

6. The factors determining the intimal hæmorrhage are probably weakening of the sinusoidal wall by toxic action and transient raised intraluminal pressure induced by exertion or emotion. In such cases superimposed intravascular thrombosis may be delayed until a subsequent period of bodily or mental rest.

I wish to thank Professor Biggart for his help, advice and encouragement during the course of this work and in the preparation of this paper. Mr D. McA. Mehaffey was responsible for the photography.

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THE ESSENTIAL IDENTITY OF THE KLIPPEL-FEIL SYNDROME AND INIENCEPHALY

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(PLATE VI)

THE deformities known as the Klippel-Feil syndrome and iniencephaly are very rare and anatomical descriptions are few and unsatisfactory. The conditions have been regarded as distinct

The *Klippel Feil syndrome* or congenital brevicolis consists of shortness of the neck, a low hair line posteriorly and limitation of movements of the head, associated with fusion of cervical vertebrae and sometimes also of upper thoracic vertebrae. Since the original publication of Klippel and Feil (1912), cases have been described which have the same anatomical lesion but differ in being without any external manifestation and many additional features have been recorded. Common among the latter are webbing of the neck (pterygium colli) due to an unusual prominence of the free borders of the trapezius muscles, marked nuchal depression at the nape of the neck, congenital high scapula, winged scapula, facial asymmetry, spasm of the cervical muscles or torticollis, cervical ribs, absence or fusion of ribs, scoliosis and dorsal kyphosis. Less common congenital abnormalities are spina bifida in the lumbar or sacral region, sacralisation of the fifth lumbar vertebra, cleft palate or abnormalities of the viscera. In a minority of cases there are nervous symptoms such as bimanual synkinesia, mental deficiency, deafness and spastic paraplegia, hemiplegia or quadriplegia, in a few instances the spastic quadriplegia has developed late in life. The head is often fixed more forward than normally. In some cases it is flexed forward so that the chin rests or almost rests upon the sternum. More commonly it is slightly retroflexed so that the face looks slightly upwards. There is often slight rotation or lateral flexion of the head to one side. Some subjects are stillborn or die early in life, possibly from associated abnormalities, but in most the deformity does not affect the prospect of life and many are adults at the time of first observation. The disease has been stated to affect males and females equally but in 71 cases to which I have referred there were 49 females to 22 males. Instances have been

recorded of the deformity in brother and sister, mother and daughter, father and son, father and daughter and mother and three children.

Knowledge of the anatomical abnormality has been gained almost entirely from X-ray appearances. Necropsies have been recorded by Klippel and Feil (1912), Crouzon and Liège (1928), Feller and Sternberg (1932), Kallius (1930-31), Feil, Lebleu and Fischer (1932) and Mitchell (1934). Other important papers on the disease are those of Greig (1924), Bauman (1932), Clemmesen (1936) and Thomson (1937).

Iniiencephaly is a deformity found only in the stillborn or in infants. One infant in the literature lived for 39 hours. This is probably about as long a post-foetal life as the deformity would ever permit. Most infants are born prematurely; some of the stillborn are macerated, others fresh. The great majority are females. In 35 cases to which I have referred 32 were females and 3 males. The deformity is very characteristic (fig. 3). There is great retroflexion of the head so that the face looks upwards and forwards. The head is commonly enlarged. The neck is absent or only indicated anteriorly below the chin. The scalp becomes continuous with the skin of the lower part of the back, perhaps as far down as the sacral region. The skin of the face usually passes directly on to that of the chest. The scapulæ are pushed aside by the head and are laterally situated. The shoulders become more anterior than normally. The bony abnormality is in the occiput, the cervical spinal column and a variable length of the spine below this. In many cases there is an encephalocele at the back of the head where it joins the back, and there may be a defect in the skin covering it. Other abnormalities are very common, particularly umbilical and diaphragmatic hernia, talipes and fusion of ribs. Less commonly recorded deformities are malformations of the mouth, sacral spina bifida, sacralisation of the fifth lumbar vertebra, celosomia and hydrocephalus. Deformities which have been recorded once each are hare-lip, cleft uvula, common mesentery to the colon and small intestine, cyclopia, abnormally short umbilical cord, malformation of the kidneys, origin of gluteus maximus from the occiput, origin of external oblique from the clavicle, imperforate anus and absence of umbilical artery. Hydramnios in the mother is not uncommon, especially where there is a defect in the skin over an encephalocele. The most important descriptions of the deformity are those of Lewis (1897), Ballantyne (1904) and Abbott and Lockhart (1905).

A CASE OF KLIPPEL-FEIL SYNDROME

Clinical history

The infant was the result of a first pregnancy. The labour was rapid and the presentation vertex. The infant, a male, weighed 5 lb. 5 oz. at birth and was probably slightly premature. The right upper limb was noticed

soon after birth to be stiff and thus led to a diagnosis of cerebral hæmorrhage. Later, signs of spastic quadriplegia were observed. The infant was cyanosed and a systolic murmur was heard all over the precordium. He failed to thrive and died at 9 weeks. He was demonstrated at the Royal Society of Medicine by MacKenzie (1937 38).

Post mortem findings

Summary of necropsy (P M 261/1938) Klippel Feil deformities. Congenital maldevelopment of heart and great vessels. Old cerebral hæmorrhage. Congestion of spleen, kidneys, thyroid and pancreas. Edema and simple atrophy of liver. Congestion and areas of collapse and of desquamative catarrh in lungs. Accidental involution of the thymus. Wasted infant.

Weight: body 2127 g, liver 71 g, kidneys 14 g, suprarenals 2.1 g, spleen 7 g, thyroid 0.85 g, thymus 0.8 g, pancreas 2.85 g, testes 0.55 g, pituitary 0.075 g, brain 404 g. Length of body 47 cm.

External Klippel Feil deformities. The neck was very short and the hair line reached at the back the level of the first dorsal spine. The head was flexed backwards and bent very slightly over to the right, the body of the lower jaw being horizontal. Movement of the head was greatly limited in all directions. There was conspicuous webbing of the skin at the sides of the neck, due to the outer borders of the trapezius muscles passing almost directly from the occiput to the outer ends of the spines of the scapulæ. The scapulæ were winged.

Klippel Feil deformities of bones and brain. The posterior cranial fossa was small and flattened and the left lateral sinus appeared abnormally large (1.3 × 0.8 cm in largest cross section). The foramen magnum was enlarged and oval (3.4 cm in sagittal diameter × 2.3 cm) and reached backwards to the external occipital protuberance (fig 1). The atlas was represented by an anterior arch and a lateral mass on each side, the posterior arch being absent. Each lateral mass had an inferior and superior articular surface and a transverse process with a foramen for the vertebral artery. The lateral part of each lateral mass and part of their transverse processes were ossified, the remainder of the atlas was cartilaginous. The joint capsules between the atlas and the occiput were thick and permitted no movement of the joints. The left inferior articular surface was directed downwards and reached to 0.2 cm from the midline. The right inferior articular surface, however, was directed downwards and inwards, reached to 0.4 cm from the midline and lay at a level 0.2 cm below that of the left. There were four partly ossified cervical vertebral bodies (fig 2). The lower three were normal in shape and could be definitely identified as the fifth, sixth and seventh. The uppermost was abnormal in shape being 0.8 cm high on the left and 0.5 cm on the right. It contained three separate bony centres of different sizes. The largest lay on the left and showed two notches in its outer border. It probably represented the fused lateral centres of the third and fourth cervical bodies. The smallest lay just to the right of the midline posterior to and between the upper parts of the two larger centres. It probably represented the displaced right lateral centre for the second cervical body. That this uppermost body certainly represented the second, third and fourth bodies was shown by its carrying the corresponding pedicles (see below). Its right superior articular surface was displaced to articulate with the displaced right inferior surface of the atlas. The tilting of the head to the right probably depended upon the displacement to the midline of the right centre of the second cervical vertebral body having made this abnormal uppermost body shorter on the right side than on the left. Six pedicles

were present on either side in the cervical region. The first, second and third on either side were attached to the abnormally shaped uppermost body and represented the pedicles of the second, third and fourth vertebrae. The fourth, fifth and sixth pedicles were attached respectively to the normally shaped second, third and fourth bodies and undoubtedly represented the pedicles of the fifth, sixth and seventh vertebrae. Transverse processes with foramina for the vertebral artery were present on either side. Those on the right were crowded together because of the tilting of the head to the right and occupied a vertical length of 0.8 cm. while the left occupied 1.4 cm. Cervical laminae were present but fusion had occurred with reduction in number to two right and four left. The first left lamina arose from the first pedicle, the second from the second and third pedicles, the third from the fourth pedicle and the fourth from the fifth and sixth pedicles. The first lamina belonged therefore to the second cervical vertebra, the second to the third and fourth vertebrae, the third to the fifth vertebra and the fourth to the sixth and seventh vertebrae. The first right lamina arose from the first, second, third and fourth pedicles and formed a plate of bone. The second arose from the fifth and sixth pedicles. The first right lamina belonged therefore to the second, third, fourth and fifth cervical vertebrae and the second to the sixth and seventh vertebrae. The laminae on either side were closely bound together by fibrous tissue and were directed downwards, backwards and inwards. The upper border of the first right lamina was directed downwards more sharply than that of the left. The posterior ends of the laminae did not fuse with those of the other side and so a posterior cervical spina bifida was present. The ends on either side were covered with cartilages which fused to form a bar, and each bar joined below in the midline its fellow of the opposite side and a mass of cartilage representing the fused spines of the upper three thoracic vertebrae. The lateral and posterior borders of the foramen magnum were closely united by fibrous tissue to the upper borders of the first laminae, the cartilaginous bars uniting on each side the posterior ends of the laminae. This close application of the foramen magnum to the borders of the spina bifida accounted for the backward tilting of the head.

The body of the third thoracic vertebra had two centres of equal size (fig. 2). The bodies of the first, second and fourth thoracic vertebrae had grooves in the middle of their upper, lower and posterior surfaces, imperfectly dividing them vertically into two.

In the cervical and upper half of the thoracic spine no intervertebral discs could be recognised. The cartilages between the bodies were white and firm. In the lower thoracic and lumbar spine intervertebral discs were present, the centres of the cartilages between the bodies being grey and pulpy. Spina bifida occulta was present in the region of the lower three sacral vertebrae.

The posterior angles of the upper four ribs were more acute than normally, especially on the left side, and the parts of the ribs anterior to the posterior angles were almost straight. The first and second left ribs were fused except at the posterior angle, where a fissure 0.5 cm. long separated them. The intercostal space between the first and second right ribs was unusually wide (0.5 cm. wide). The bony part of the fourth right rib ended 1.2 cm. behind the line of the costo-chondral junctions of the other right ribs, and the bone passed into a thin atrophic costal cartilage without any of the swelling present at the other costo-chondral junctions.

The cerebellum was flattened in correspondence with the flattening of the posterior fossa. A little of the under surface of the left hemisphere protruded through the foramen magnum and an antero-posterior groove, 2.5 cm.

KLIPPEL FEIL SYNDROME AND MENINGOCEPHALY



FIG. 1 — Klippel Feil syndrome. Partly dissected skeleton with skull and lateral masses of the atlas separated from the remainder so as to reveal the posterior spina bifida and elongated medulla with its prominent restiform bodies bounding the fourth ventricle.



FIG. 2 — Klippel Feil syndrome. Radiograph of partly dissected skeleton with occiput forcibly elevated to show the state of the cervical column. Pin in first thoracic vertebral body. A right lateral mass of the atlas. B first right cervical lamina. C second right cervical lamina. Two bony centres in the third thoracic vertebral body and incomplete fusion of two centres in the bodies of the first, second and fourth thoracic vertebrae.



from the midline, had been formed upon it. The medulla was considerably elongated (3 cm. long); all but its uppermost part lay behind the cervical vertebral bodies, while its lower end reached the lower border of the first dorsal vertebral body (fig. 1). The fourth ventricle was correspondingly elongated and was bounded laterally by prominent restiform bodies.

Cardiovascular developmental abnormalities. The foramen ovale was patent (0.7 cm. diameter). There was a patency (0.35 cm. diameter) in the interventricular septum immediately anterior to the pars membranacea septi. The aorta arose from the right ventricle and lay in the usual position of the pulmonary artery. The pulmonary artery was thin-walled and lay in the usual position of the ascending aorta, but continued as a canal (0.3 cm. diameter) which passed obliquely through the upper and anterior part of the interventricular septum just below the patency to open into the right ventricle without communicating with the left. The ductus arteriosus was very small but patent (0.15 cm. diameter).

Old hæmorrhages in the brain. There was a firm white area (1 cm. diameter) of old hæmorrhage, mottled with rusty areas and flecked with opaque yellow lipoid, beneath the ependyma over the outer and posterior aspect of the anterior horn of the left lateral ventricle, with two extensions (1 cm. long) into the subjacent white matter. A second area of old hæmorrhage (1.5 × 0.5 cm.) lay in the subcortical white matter of the upper and posterior part of the left parietal region and was firm, white and flecked with yellow lipoid. A third similar area (1 × 0.4 cm.) lay beneath the ependyma of the descending horn of the left lateral ventricle.

Microscopically the first area showed proliferation of fibrillary astrocytes, some of which were rounded and had abundant cytoplasm. The ependymal cells over the area were elongated by processes containing glial fibrils which extended into it. There were numerous cholesterol-ester phagocytes in the area, partly scattered, forming small groups, partly producing a large streak. In the streak there was some intra- and extra-cellular amorphous hæmatoidin and granular hæmosiderin. Some of the hæmosiderin granules were stained dark brown or almost black by Ehrlich's hæmatoxylin owing to the presence of traces of ferrous iron mixed with the ferric. In the areas of gliosis there were numerous granules, some fused to form small plaques up to 12 μ diameter, of material stained deep blue-black by Ehrlich's hæmatoxylin, giving Kossa's reaction, staining strongly for ferric iron and slightly for ferrous and showing the presence of calcium by production of gypsum crystals with sulphuric acid. Some of these granules were in cells, apparently degenerated phagocytes, others were free. In frozen sections crystals of hæmoglobin were present in the streak of phagocytes.

Comment

The case presents the classical features of the Klippel-Feil syndrome in the shortening of the neck, low hair-line and limitation of movements of the head. The essential bony abnormalities appeared to be as follows: failure of union in the midline of bilaterally formed centres in the second, third and fourth cervical vertebral bodies; fusion of most of these lateral centres on each side; failure of union, or incomplete union, of bilaterally formed centres in the first, second, third and fourth thoracic vertebral bodies; absence of intervertebral discs between the cervical and upper six thoracic vertebral bodies; posterior cervical spina bifida;

fusion of several cervical laminae. There was an apparent numerical reduction of laminae and bodies, but it was not real and was only produced by fusion. Bardeen (1910) stated that in the normal development of the vertebrae the cartilaginous bodies were formed in two centres of chondrification which soon united. Usually there was only one centre of ossification, but occasionally two. I do not consider, therefore, the formation of the bodies from two centres of ossification to be abnormal, but their failure of union, or imperfect union, by the time of infancy is certainly abnormal.

It is difficult to compare the bony lesions in my case with those described in the literature, whether as revealed by X-rays or at the rare necropsies. Posterior spina bifida appears to be constant, although in a few cases it was not seen in radiographs. In a few cases it appeared in radiographs to be limited to the lower cervical region, but in all others it affected the atlas and a variable number of subjacent vertebrae. I do not know of cases showing anterior spina bifida, but there is no reason why this should not occasionally occur from non-union of the bilaterally formed centres of chondrification and consequent complete separation of two centres of ossification. Fusion of vertebrae is constant but variable in degree. With fusion of only two vertebrae or parts of two vertebrae, it is quite possible that posterior spina bifida would be absent, but in such cases the malformation would be too slight to show the Klippel-Feil syndrome. The degree of bony fusion undoubtedly varies with age as well as with severity of the abnormality. In many of the examples in adults the cervical and many of the upper thoracic vertebrae have been fused into one mass in which separate vertebrae could be recognised only by the transverse processes or other parts of the arches. In my case, if the subject had lived, not only the cartilaginous bars joining on either side the posterior free ends of the cervical laminae, but the cartilage between the bony centres of the cervical and upper thoracic bodies would have become ossified, the latter because of the absence of intervertebral discs. A single bony mass would thus have been formed. The transverse processes, pedicles and reduced number of cervical laminae would still have been distinct and recognisable, however, because they had been separated by connective tissue and not joined by cartilage. The atlas would likewise have remained separate, both from the occiput and from the remainder of the cervical column. An appearance would have resulted like that in Klippel and Feil's case and case 6 of Feller and Sternberg, both of which were adults. The condition in Feller and Sternberg's case 3, an infant, resembles that in mine. They called the upper of the three cervical bodies present the atlas, but since the atlas has no body, their upper body probably represented the axis and perhaps also the body of the third cervical vertebra. In most cases

the atlas has been separate from the occiput. In a few cases they have been fused, as shown at necropsy (by Mitchell) or by X rays.

Evidence in the literature of the non union or incomplete union of bilaterally formed centres is scanty. In Feller and Sternberg's case 6 the fifth thoracic body showed a ridge suggesting an origin from two centres and in their case 4 the first thoracic body was divided vertically into two separate parts, while the third thoracic was similarly divided into two parts joined by a narrow bridge.

Evidence of true numerical reduction of vertebrae or parts of vertebrae by congenital absence is present only in a few cases.

In Feller and Sternberg's case 3 the second thoracic body was represented by one lateral nucleus. In their case 4 the second thoracic vertebral body was represented by one lateral nucleus, which was joined to the body below. A contralateral nucleus had probably failed to appear in these vertebrae. A similar condition has been shown by X rays, for instance by Odén (1934) in the seventh cervical vertebra, and by Hadley (1940) in the sixth cervical.

In my case the foramen magnum was slightly enlarged and its posterior and lateral borders were bound by fibrous tissue to the borders of the spina bifida, thus accounting for the slight retroflexion of the head. A similar occurrence has been reported in several cases. In other cases, especially those with forward flexion of the head, the gap over the spina bifida may have been bridged by membranes and not by the occipital bone. Feil, Lebleu and Fischer described a case with a hernia through the posterior spina bifida, apparently an encephalocele. This appears to be unique.

Deformities of the central nervous system have not been recorded in the literature. One would expect the prolapse and elongation of the medulla seen in my case to occur frequently. Some of the nervous symptoms that have been described, such as quadriplegia, paraplegia or hemiplegia, may have been the result of this condition. In my case the cause of the quadriplegia is open to doubt, because there were old hæmorrhages in the brain. The absence of hydrocephalus showed that the deformity of the medulla had produced no obstruction to the flow of cerebro spinal fluid.

A CASE OF INIENCEPHALY

Obstetric history

The mother was a primipara and developed hydramnios at 32 weeks. X rays then showed acute lordosis of the foetal cervical spinal column. Pyelitis of pregnancy developed. Labour was normal. The foetus, a male, was stillborn.

Post mortem findings

Summary of necropsy (P.M. 50/1940) Iniencephaly. Congenital maldevelopment of heart and vessels, mouth, lungs, mesenteric attachments, intestines, kidneys, urinary bladder, œsophagus, pancreas and thymus.

fusion of several cervical laminæ. There was an apparent numerical reduction of laminæ and bodies, but it was not real and was only produced by fusion. Bardeen (1910) stated that in the normal development of the vertebræ the cartilaginous bodies were formed in two centres of chondrification which soon united. Usually there was only one centre of ossification, but occasionally two. I do not consider, therefore, the formation of the bodies from two centres of ossification to be abnormal, but their failure of union, or imperfect union, by the time of infancy is certainly abnormal.

It is difficult to compare the bony lesions in my case with those described in the literature, whether as revealed by X-rays or at the rare necropsies. Posterior spina bifida appears to be constant, although in a few cases it was not seen in radiographs. In a few cases it appeared in radiographs to be limited to the lower cervical region, but in all others it affected the atlas and a variable number of subjacent vertebræ. I do not know of cases showing anterior spina bifida, but there is no reason why this should not occasionally occur from non-union of the bilaterally formed centres of chondrification and consequent complete separation of two centres of ossification. Fusion of vertebræ is constant but variable in degree. With fusion of only two vertebræ or parts of two vertebræ, it is quite possible that posterior spina bifida would be absent, but in such cases the malformation would be too slight to show the Klippel-Feil syndrome. The degree of bony fusion undoubtedly varies with age as well as with severity of the abnormality. In many of the examples in adults the cervical and many of the upper thoracic vertebræ have been fused into one mass in which separate vertebræ could be recognised only by the transverse processes or other parts of the arches. In my case, if the subject had lived, not only the cartilaginous bars joining on either side the posterior free ends of the cervical laminæ, but the cartilage between the bony centres of the cervical and upper thoracic bodies would have become ossified, the latter because of the absence of intervertebral discs. A single bony mass would thus have been formed. The transverse processes, pedicles and reduced number of cervical laminæ would still have been distinct and recognisable, however, because they had been separated by connective tissue and not joined by cartilage. The atlas would likewise have remained separate, both from the occiput and from the remainder of the cervical column. An appearance would have resulted like that in Klippel and Feil's case and case 6 of Feller and Sternberg, both of which were adults. The condition in Feller and Sternberg's case 3, an infant, resembles that in mine. They called the upper of the three cervical bodies present the atlas, but since the atlas has no body, their upper body probably represented the axis and perhaps also the body of the third cervical vertebra. In most cases

thoracic arches and bodies and numbered from above down (fig 4) The *left cervico thoracic arches* were nine in number 1, 1 cm long, probably belonged to the second cervical vertebra, 2, a triangular plate of bone 1 cm long with three pedicles, probably belonged to the third, fourth and fifth cervical vertebrae, 3 and 4, 0.7 cm long, probably belonged to the sixth and seventh cervical vertebrae, 5, a triangular plate of bone with several pedicles, corresponded to the upper five ribs and so belonged to the upper five thoracic vertebrae, 6, 7, 8 and 9 corresponded to ribs and vertebrae of similar numerical value. The *right cervico thoracic arches* were twelve in number 1-4 were similar to the left, 5-12 belonged to the second to the ninth thoracic vertebrae inclusive and increased in size from above downwards, 5 being very small and rudimentary. No right arch corresponding to the first thoracic vertebra was found. Transverse processes were absent on the cervico thoracic arches 1-4, rudimentary on those representing the upper thoracic vertebral arches

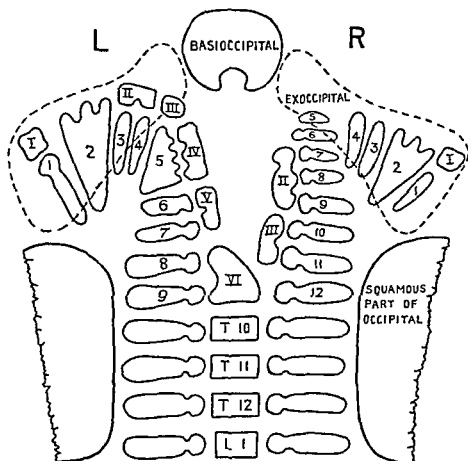


FIG 4—Iniencephaly. Diagrammatic representation of the bony deformity. The abnormal cervico thoracic vertebral arches are numbered 1-9 on the left side and 1-12 on the right; bodies are numbered I-VI on the left side and I-III on the right.

and well developed on the remainder. The *left cervico thoracic bodies* were six in number. I, a cubical bone with the corners rounded off, was applied to the upper end of the left cervico thoracic arch 1 and had an articular surface on its upper aspect corresponding with another on the under surface of the occipital, it probably represented the left lateral centre of the second cervical vertebra (the atlas being absent), or possibly the lateral mass of the atlas because of its articulation with the exoccipital, II, a rectangular bone with the corners rounded off and a groove in the middle of its under surface, lay above arches 3 and 4 and represented the fused left centres of two cervical bodies, probably the sixth and seventh, III, a small rectangular bone, lay adjacent to the upper part of the cervico thoracic arch 5 and probably

represented the left centre of either the first or second thoracic vertebra; IV, a long rectangular bone with two grooves in its surface, lay adjacent to the inner end of the cervico-thoracic arch 5; it probably represented the fused left centres of three upper thoracic vertebral bodies, possibly the third, fourth and fifth; V, a bone shaped like body II, lay opposite the cervico-thoracic arches 6 and 7 and probably represented the fused left centres of the bodies of the sixth and seventh thoracic vertebrae; VI lay in the midline; its high left side was notched in the centre and corresponded in position to the left cervico-thoracic arches 8 and 9, while its low right side corresponded to the right arch 12, the bone being the ninth thoracic body fused with the left centre of the eighth. The *right cervico-thoracic bodies* were three in number: I was similar to that on the left side; II lay adjacent to the inner ends of the right cervico-thoracic arches 7, 8 and 9, was divided into three indistinct parts by grooves and probably represented the fused right centres of the fourth, fifth and sixth thoracic vertebral bodies; III, a rectangular bone with a groove in the centre of its outer surface, lay adjacent to the inner ends of the right cervico-thoracic arches 10 and 11 and represented the fused right centres of the seventh and eighth thoracic bodies.

The cervico-thoracic bodies and arches were *abnormally orientated*. Those representing the first to the ninth thoracic vertebrae diverged from the midline and bent forwards, both the divergence and forward bend increasing from below upwards. The upper ends of the bifid thoracic column reached the under surfaces of the antero-mesial ends of the exoccipitals close to the basioccipital and thus bordered with the occipital a hole in the anterior wall of the spinal column. The bodies and arches that represented the cervical vertebrae were bent backwards and outwards on each side so that the arches lay almost vertically and their upper ends ran in a line along the under surfaces of the exoccipitals to the articulation between the cervico-thoracic bodies I and the postero-lateral ends of the exoccipitals. This line made an angle of between 70° and 80° with the thoracic column on either side. A small recess was formed on each side underneath the exoccipitals by the abnormally orientated arches.

There were twelve ribs on either side. The bony parts of the first ribs were very short (1.5 cm. long), that of the left being very thin (1 mm. in diameter). The first left rib was fused with the second near the junction of the latter's anterior and middle thirds. The upper ribs were much squashed together at their posterior ends. The clavicles were flattened at their centres. The lower jaw was delicate and deformed. Its angle was very obtuse (about 140°) so that the rami were almost in line with the body. The external surface and the lower border of each lateral half of the body ran without any curve to meet at the symphysis at an angle of about 60° . The lower border was sharp. The internal surfaces were traversed by prominent internal oblique lines. The upper border, however, was curved like a horseshoe. The external surfaces sloped upwards and inwards to meet it. It was much shorter than the lower border, so that the symphysis sloped upwards and backwards at an angle of about 50° , making the chin extremely prominent. The symphysis was marked externally by an unusually prominent ridge, which ended inferiorly in a sharp-pointed, projecting mental protuberance without mental tubercles.

The brain was very deformed. The corpus callosum and the fornices were absent and the hemispheres were united by a transparent film, apparently of leptomeninges. The lateral ventricles were greatly dilated and showed no differentiation into horns, while their ependyma was mottled with hæmorrhages and showed a number of grey smooth bosses up to 5×4 mm. in diameter. There were no foramina of Monro. The site of the third ventricle

was occupied by a solid mass of grey matter continuous below with a similar mass occupying the mid brain. There was no trace of an aqueduct. There were no crura cerebri. There was an acute ventral kinking of the pons and medulla, which together formed a bulbous mass 1.8 cm in diameter, the apex lying opposite the hole in the anterior wall of the spinal column. The mass was flanked on either side by symmetrical protuberances of medulla, 1 cm long, which lay in the lateral recesses beneath the exoccipitals. The cerebellar hemispheres were rudimentary (about 1 cm in diameter) and lay on either side between the brain stem and the medullary protuberances. There was a cavity, the fourth ventricle, in the brain stem at this level. This cavity rapidly tapered below the level of the protuberances, and the tissue roofing it tapered to a spur, 0.5 cm long, which projected over the lumbar enlargement.

Developmental abnormalities of heart and vessels (including vessels in abdomen) There was a patent foramen ovale 6 mm in diameter and a patency (12×7 mm) in the interventricular septum. The artery arising from the right ventricle in the usual site of the pulmonary artery gave off two coronary arteries from sinuses of Valsalva and, after a vertical course of 1.5 cm, divided into three branches, the right subclavian and the common carotids. The bifurcations of the common carotids were unusually low, being opposite the lower border of the cricoid. The artery arising from the left ventricle in the usual site of the aorta gave off the right and left pulmonary arteries 0.8 cm above the valve and then arched backwards and downwards to become the descending thoracic aorta, from which the left subclavian arose 2.5 cm beyond the valve. A small ductus arteriosus, 2 mm in diameter, passed from the descending aorta, 1.8 cm beyond the valve, to the artery arising from the right ventricle. The superior vena cava passed upwards from the heart in a sheath of pericardium through the thoracic cavity and not in the mediastinum. The intrathoracic inferior vena cava was formed by the hepatic veins and the ductus venosus. The intra abdominal inferior vena cava passed upwards into the thorax on the right of the aorta and arched forwards above the right and accessory lungs to join the superior vena cava. The left common and internal iliac arteries were 10 mm, the right 5 mm in circumference. The right hypogastric and one umbilical artery were absent.

Other developmental abnormalities in the thorax The lungs were small ($5 \times 2.5 \times 1$ cm) and the right had only two lobes. An accessory right lung lay between the superior vena cava and the arterial trunk arising from the right ventricle. It was joined to the right upper lobe along the latter's mesial surface. Its bronchus joined the trachea immediately above the right main bronchus. The parietal pericardium was absent except on the right side, where it passed upwards to form a sheath to the superior vena cava.

Developmental abnormalities in mouth and neck tissues The right angle of the mouth was 1.2 cm from the midline while the left was 1.7 cm. A fold of buccal mucosa 6 mm wide and 5 mm high passed from the soft palate to the left angle of the mouth, and the lips united with the fold and not with each other. There was no intrathoracic thymus. Serial sections of the neck organs showed that thymus III was hypoplastic and represented by a rounded mass, 2.5×1.5 cm, divided into two lobes, which lay just above the sternum in the midline. There were two small accessory right lower parathyroids and one accessory left lower, giving seven parathyroids in all. One right accessory parathyroid was fused with a small accessory mass of thymus III, a thymus lobule III. The main left lower parathyroid was fused in part with trabeculae of thyroid tissue. The upper part of the thyroglossal duct was persistent and ran from the foramen cecum to the region

above and in front of the hyoid. The upper part was lined with squamous, the lower with columnar epithelium. There was a small mass of accessory thyroid tissue just in front of the middle of the hyoid and just below the end of the duct. The oesophagus was very short and wide (3 cm. long and 3.5 cm. in circumference) and joined the stomach without any alteration of circumference. Its posterior wall was adherent to the red vascular connective tissue occupying the hole in the anterior wall of the spinal column. There were a few varices in the submucosa.

Developmental abnormalities in the abdomen. There were thirteen accessory spleens, the largest 4 mm. in diameter, grouped like a bunch of grapes and attached to the splenic hilum. The left kidney was larger than the right and was converted into a reniform mass of thin-walled cysts (the largest 2 × 1 cm.). There were a few submiliary areas of hyaline cartilage in the remnants of normal kidney tissue or between the cysts. The pancreas, 9 cm. long, was in two parts. One was attached along the lower part of the greater curvature of the stomach, the other ran horizontally below it from the main spleen; the two were united for a short distance near the duodenum. The small and large intestines had a common mesentery. The duodenum had a short mesentery. A Meckel's diverticulum, 11 cm. long, was present on the ileum 24 cm. above the cæcum. There was a cyst, 5 × 3 mm., in the peritoneal cavity, attached by a fibro-vascular strand, 4 cm. long by 0.5 cm. in diameter, to the inner aspect of the umbilicus. Its wall was similar to that of the normal small intestine and it contained mucus and desquamated cells. It was apparently a segment of the vitelline duct. The urinary bladder was pear-shaped, with a tapering end reaching almost to the umbilicus. There was a urachal cyst, 1.5 × 0.3 cm., with a wall similar to that of the normal bladder, in a fibrous cord connecting the bladder to the umbilicus. A hernial sac 2.5 cm. long and 1.5 cm. in diameter passed upwards behind the heart through the posterior part of the middle of the diaphragm. It had a thin peritoneal wall and contained the greater part of the stomach, the spleens and the pancreas.

Comment

The case shows the typical body deformity of iniencephaly. The essential bony abnormalities appeared to be as follows: wide separation of bilaterally formed bodies of cervical and upper thoracic vertebræ, with the formation of an anterior spina bifida; congenital absence of one or both lateral halves of many cervical bodies; fusion of a variable number of the cervical and upper thoracic bodies on either side; marked posterior spina bifida in the cervical and thoracic regions; fusion of several cervical or thoracic laminae; cleavage of the squamous part of the occipital bone. The atlas appeared to be absent. The bones which articulated with the occipitals had the shape of vertebral bodies rather than of the lateral masses of the atlas and they were closely related to the first arches representing those of the axis. The bones probably represented, therefore, the lateral centres of the body of the axis. There was a considerable numerical reduction of vertebral bodies in the cervical region from congenital absence, but the arches, although sometimes fused, were all present except the first right thoracic and those of the atlas. The marked cervico-thoracic lordosis

was probably secondary to the great deformity of the spine and the great retroflexion of the head. It was formed by the thoracic representatives of the abnormal cervico thoracic vertebræ on each side being deflected outwards and forwards increasingly from below upwards, while the cervical representatives bent outwards and backwards from below upwards. The anterior spina bifida probably resulted from non union of the bilaterally formed centres of chondrification so that, when the latter were ossified, the two bony centres in each body remained unconnected.

From the cases in the literature one can assume that posterior spina bifida is constant, while anterior spina bifida is very common but probably not constant. The posterior spina bifida involves the whole cervical region, a variable number of thoracic vertebræ and sometimes also the lumbar region. Feller and Sternberg's cases 1, 2 and 5 were regarded as cases of Klippel Feil syndrome but they were undoubtedly cases of miencephaly of a slightly milder degree than in my case. Anterior spina bifida was present in their cases 1 and 2 but apparently not in case 5. It was also absent in the case of Vallois and Vallois (1914). Whether non-union of bilaterally formed centres in the bodies, fusion of these centres on either side and congenital absence of some centres are constant cannot be ascertained from cases in the literature, but they were present in Feller and Sternberg's three cases. Marked cervico thoracic lordosis is reported by most authors and is probably constant. The squamous part of the occipital bone is usually reported as being in two separate parts, with the same relations as in my case. In Vallois and Vallois's case the foramen magnum was formed but was enormous. In their figure the squamous part of the occipital appears as if it were in two parts which met in a suture behind the foramen magnum. An encephalocele herniated through a cleft squamous part of the occipital or through an enlarged foramen magnum, as in Vallois and Vallois's case, is very common. Lewis divided the condition into miencephalus apertus and miencephalus clausus according to whether or not there was an encephalocele, and he subdivided the former according to whether it was small or large. The skin covering the encephalocele is in some cases defective. Lewis (1897), Ballantyne (1904) and Howkins and Lawrie (1939) described cases of anencephaly or acrania with great retroflexion of the head resembling that in miencephaly. This is probably due to an associated abnormality in the cervical spine similar to that in miencephaly.

In two cases mentioned by Lewis there was a deformity of the mouth, but it was not quite the same as in my case. In another case described by him the jaw in his drawing shows a deformity not unlike that in my case. Hayes (1921-22) described a case in which the thymus formed a spherical mass in the neck above the sternum, as in my case. This condition is not constant because in Ballantyne's

three cases drawings show the thymus in its normal position in the thorax.

The oedema which occurred in my case was probably cardiac in origin. Oedema of the neck was present in a case of Lewis's and was probably cardiac or mechanical in origin, but he does not mention whether congenital morbus cordis was present or not.

Hydrocephalus has been reported in only a minority of cases but it may have been of more frequent occurrence. It might be expected to be the result of deformity of the brain secondary to that of the bones but in my case it was due to an independent abnormality in the brain—absence of the aqueduct of Sylvius.

COMPARISON OF THE DEFORMITIES OF THE KLIPPEL-FEIL SYNDROME AND INIENCEPHALY

Both diseases show non-union of bilaterally formed centres of ossification in the bodies of the cervical vertebræ and of a variable number of subjacent vertebræ, and fusion on each side of some of these centres. Posterior spina bifida and fusion of some laminae are present in both. In iniencephaly probably constantly and in the Klippel-Feil syndrome probably rarely there is a congenital absence of centres of chondrification for some bodies on one or both sides and so an absence of bony centres. In iniencephaly there may also be congenital absence of arches, but the number absent is not so great as that of the bodies. Further, in iniencephaly there is commonly in addition an anterior spina bifida and a cleavage of the squamous part of the occipital bone. The former results from non-union of the bilateral centres of chondrification in the bodies. The latter is an extension of the bifid state of the arches of the spine to the occiput, and it is this that accounts for the frequency of posterior encephalocele in iniencephaly.

In at least most cases of the Klippel-Feil syndrome and probably in all cases of iniencephaly the borders of the foramen magnum, or in the case of iniencephaly the inner borders of the cleft squamous part of the occipital, are closely applied to the borders of the spina bifida. This accounts for the great retroflexion of the head in iniencephaly and the slight retroflexion common in the Klippel-Feil syndrome.

In both conditions other congenital abnormalities are frequent, and many are common to both, especially deformities of the ribs and spina bifida in the lumbo-sacral region. Congenital morbus cordis was common to my cases.

It is apparent that iniencephaly is a more severe form of the same deformity as that in the Klippel-Feil syndrome and is severe enough to prevent the subject living more than a few hours. In the Klippel-Feil syndrome the patient does not die of the direct effects of the deformity but from either associated abnormalities,

indirect effects of the deformity or independent causes. In the spine in adult life in the Klippel Feil syndrome the original state of the bodies of the vertebrae is masked by progressive ossification fusing the two bony centres present in many of the bodies not separated by intervertebral discs.

In support of my belief that the two deformities are essentially the same is the recording by Feller and Sternberg of 3 undoubted cases of iniencephaly as cases of the Klippel-Feil syndrome. The differences found at necropsy between the deformities in these 3 cases and in their 3 cases of true Klippel Feil syndrome must have appeared so slight that they did not consider the possibility of two separate diseases. They did not consider the question of iniencephaly at all.

SUMMARY

An example of the Klippel Feil syndrome and of iniencephaly are described, with a detailed report of the bony deformities found at necropsy in each. From the similarity of the changes in these two cases and in cases in the literature described under one or other name it is concluded that the Klippel Feil deformity is a mild form of the deformity characteristic of iniencephaly.

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THE DISTRIBUTION OF FATTY CHANGE IN THE KIDNEYS AND SOME FACTORS INFLUENCING ITS PRODUCTION

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(PLATE VII)

PART I

In a previous paper (Dible and Hay, 1940), it has been shown that the fat which accumulates in the kidney when animals with sufficient fat reserves are subjected to starvation is localised principally in the zone of Henle's tubules

The localisation of the fatty change in heart muscle in the inner wall of the ventricles and papillary muscles enabled us to make separate estimations of the fatty and non fatty areas in the same heart, to demonstrate quantitatively the extent of the fatty infiltration and to dispose, in this instance at any rate, of the doctrine of phanerosis as the explanation of this change (Dible, 1934)

In the work now described we have carried out analogous investigations upon the kidney and by separating the cortex (which usually showed no visible fat) from the medulla (which contained the fatty zone), we have been able to estimate the fat content of those portions of the organ separately and to correlate the extent of the increase with the degree of histological change

TECHNIQUE

The animals used were rabbits of about 2000 g, all of which were starved for a period of six days. In previous experiments this had been shown to give the desired result. Water was not withheld. At the end of the period of fast the animals were killed and examined. The kidneys were carefully dissected, the capsule and pelvic fat being removed as completely as possible. The organ was then sliced and the fatty and non fatty parts of the kidney were separated. In series I, this consisted of a dissection of the cortex from the medulla, which contained most of the visible fat in its outer zone. The fat content of the two parts was estimated separately. In the experiments in series II a more complete separation was effected, the kidney being divided into three parts: cortex, outer medullary zone (outer and inner stripes of Peter) and inner medullary zone. The concentration of fat under the

* Holding a British Council scholarship.

conditions of these experiments in the outer medullary zone has been described and figured by Dible and Hay in this *Journal* (1940, li, 1; figs. 1 and 4). In neither series of experiments is it claimed that the separation of the kidney into fatty and non-fatty zones was anything more than approximate, but it seemed to us sufficiently effective to be certain to yield positive results in the chemical estimations provided the visible fatty changes were of the nature of an absolute increase.

A rough comparative measure of the quantity of storage fat in the animals' bodies was made by taking the combined weight of the omental and paravertebral fat deposits in each instance. The fats were estimated by the method of Leathes and Raper and the iodine values by Dam's method.

RESULTS

Series I

These are given in tables I and II, comprising control animals and animals subjected to starvation. These results show that

TABLE I

Fat content of kidneys of control rabbits (series I). Cortex and medulla were examined separately

No.	Body weight (g.)	Kidney				Body adipose tissue (g.)
		Cortex		Medulla		
		Fat content (percentage of moist wt.)	Iodine value	Fat content (percentage of moist wt.)	Iodine value	
PS 2	1230	2.38	93	1.48	102	0
F 1	2100	2.33	84	1.31	89	10
F 2	1300	2.58	108	1.47	98	0
F 3	1350	2.42	106	1.49	89	0
F 4	2650	2.63	104	1.50	97	100
F 5	1860	2.56	101	1.51	96	10
Means	2098	2.48	99.3	1.46	95	...

If we include the figures from table III the mean cortical fat content and iodine value would be 2.64 ± 0.153 and 102 respectively.

normally there is a great deal of difference between the invisible cell-fat content of the cortex and medulla, the respective figures for our animals being about 2.6 and 1.5 per cent. of the moist weight. Where visible fatty change was produced by starvation a slight increase was found in the cortical fat, the mean for which was 2.98 as against 2.64 in the control (see footnote, table I), an increase of 13 per cent. and a much greater increase in the medulla, the relative figures being 2.24 and 1.46 per cent., an increase of 53.5 per cent. These figures are considerably in excess of most of those obtained by Dible and Hay, who found a mean increase of

TABLE II
Fat content of kidneys and liver of rabbits starved for 6 days (series I) Cortex and medulla were examined separately

No	Initial body weight (g)	Final body weight (g)	Percentage of body weight lost	Kidney						Liver			Body adipose tissue (g)
				Cortex			Medulla			Fat content (percentage of moist wt)	Iodine value	Microscopic fat content	
				Fat content (percentage of moist wt)	Iodine value	Microscopic fat content	Fat content (percentage of moist wt)	Iodine value	Microscopic fat content				
D 1	2420	2140	11.6	*R 2.78 L 2.80	98 100	—	1.82 1.77	87 88	±			±	9.6
D 2	2500	1850	26.0	R 3.15 L 3.14	102 103	—	2.38 2.28	94 92	++	4.53	99	+	0
D 3	3450	2620	24.1	R 2.92 L 3.07	100 98	—	2.28 2.34	84 87	++	9.23	80	+++	25.0
D 4	2300	1750	23.9	R 3.06 L 3.05	94 92	—	2.35 2.63	85 84	++	5.84	89	++	17.0
D 5	2400	2000	16.7	R 2.98 L 2.88	99 101	—	2.43 2.15	90 91	++	5.40	91	+	5
Means	2614	2072	20.5	2.98	98.7		2.24	88		6.25	89.7	.	.

* R = right kidney, L = left kidney

only about 17 per cent. in their most successful experiments, when the estimations were made on the whole kidney.

Histologically there was visible fatty change to be seen in Scharlach R-stained sections in the medulla of each of the kidneys of the starved animals. No visible fat was seen in any part of the kidney in the control animals.

Series II

In these animals estimations were made upon three zones in the kidneys: (a) the cortex, (b) the outer zone of the medulla containing the portions of Henle's tubules, which showed as a rule the maximum

TABLE III

Fat content of kidneys of control animals (series II). Cortex, Henle zone and medulla were examined separately

No.	Body weight (g.)	Kidney				Body adipose tissue (g.)
		Cortex		Henle zone (H) and medulla (M)		
		Fat content (percentage of moist wt.)	Iodine value	Fat content (percentage of moist wt.)	Iodine value	
1528	2600	2.76	108	H 1.79 M 1.01	102 ...	80
1587	2690	2.76	112	H 1.73 M 0.99	97 ...	20
C 1	2770	2.75	96	H 1.92 M 1.00	91 ...	42
C 2	2300	2.71	100	H 1.72 M 1.03	85 ...	14
C 3	2450	2.91	99	H 1.96 M 1.15	92 ...	30
C 4	1920	2.71	102	H 1.72 M 0.98	103 ...	0
C 5	2430	2.67	107	H 1.82 M 1.14	100 ...	19
C 6	2320	2.73	105	H 1.92 M 1.43	85 ...	30
C 7	2650	2.74	100	H 1.79 M 0.79	97 ...	80
C 8	3000	2.54	104	H 1.75 M 1.00	89 ...	90
Means	2513	2.73 ± 0.087	103	H 1.81 ± .086 M 1.05 ± .154	94 ...	

If we include the figures from table I, the mean cortical fat content and iodine value would be 2.64 ± 0.153 and 102 respectively.

degree of fatty change (referred to as the "Henle zone") and (c) the remainder of the medulla, which was usually devoid of fatty change. In addition to the starved animals we have included in this series two other animals which died in the laboratory during our experiments and which showed fatty kidneys. The results in series II are given in gross in tables III and IV. In the analysis of these results it is legitimate to exclude no. 11, which failed to develop fatty change in its kidneys.

The mean figures for the controls and for those experimental animals in this series which developed the fatty change are as follows

	Cortex	Henle zone	Medulla
Controls	2.64	1.81	1.05
Starved animals	2.97	2.71	1.15
Percentage increase	12.5	49.7	9.2

These figures further emphasise the very different quantities of cell fat which are normal to the different zones of the kidney. In the rabbit the cortex has the highest content of essential fat, next comes the Henle zone of the medulla (the outer and inner stripes of Peter), and finally the inner zone of the medulla, which consists very largely of collecting tubules. It is, therefore, of considerable significance that the ratio of fat content between cortex and Henle zone, which is normally about 3:2, is greatly reduced in the fatty kidneys and that in some instances (animals 6, 12, 13, 16 and 1526) the percentage of fat in the Henle zone actually exceeds that in the medulla. This finding clearly points to infiltration as being the mechanism of the fatty change.

Further analysis of the results in series II

In certain of these experiments, which will be considered in the second part of this paper, a factor—abnormal temperature—the significance of which had not previously been considered, influenced the results, so that those given in table IV, which includes all the animals in this series, are not strictly comparable with one another. The animals involved were nos. D 14 to D 22. If we exclude these the corrected means are as follows

	Cortex	Henle zone	Medulla
Controls	2.64	1.81	1.05
Starved animals	3.01	2.95	1.17
Percentage increase	14.0	62.0	11.0

TABLE IV—*Fat content of kidneys and liver of experimental animals (series II). Cortex, Henle zone and medulla were examined separately*

No.	Initial body wt. (g.)	Final body wt. (g.)	Per-centage of body wt. lost.	Kidney						Liver				Body adipose tissue (g.)	Remarks		
				Cortex			Henle-zone (H) and medulla (M)			Fat content (per-centage of moist wt.)	Iodine value	Microscopic fat content	Fat content (per-centage of moist wt.)			Iodine value	Microscopic fat content
				Fat content (per-centage of moist wt.)	Iodine value	Micro-scopic fat content	Fat content (per-centage of moist wt.)	Iodine value	Microscopic fat content								
D 6	2310	1960	15.1	3.14	89	—	H 3.77 M 1.44	75	+++	+	14.01	69	+++	45	...		
D 7	2700	2150	22.9	2.85	99	—	H 2.59 M 1.14	89	++	+	4.44	93	+	4	...		
D 8	1970	1710	13.2	2.93	98	—	H 2.85 M 1.14	85	++	+	6.01	85	++	17	...		
D 9	2650	1950	26.4	2.83	105	—	H 2.13 M 1.09	99	±	±	3.74	106	±	0	...		
D 10	1090	1480	25.6	3.11	108	+	H 2.35 M 1.07	93	+	+	4.17	99	+	3	...		
D 11	2540	1880	26.0	2.61	109	—	H 1.94* M 0.96	97	—	—	2.83*	109	—	0	...		
D 12	2840	2200	22.5	3.38	88	—	H 3.41 M 0.95	74	++	+	10.56	76	+++	15	...		
D 13	2870	2250	21.6	3.45	104	++	H 3.94 M 1.01	78	+++	+	13.35	74	+++	60	...		
D 14	2720	2270	16.5	3.21	93	—	H 2.31 M 0.89	88	+	—	5.05	89	+	42	Experiment in cold weather, tem- perature about +1° to +10° C.		
D 15	2920	2100	17.8	2.85	99	—	H 2.31 M 0.89	87	+	—	5.17	96	+	20	" "		
D 16	3700	2880	22.1	2.49	92	—	H 2.53 M 1.00	85	+	—	3.25*	97	—	36	Temperature during experiment: min. 0° C., max. +10° C.		
D 17	2570	2000	22.2	2.77	96	—	H 2.03 M 1.33	93	+	—	4.27	90	+	9	Temperature during experiment: min. 0° C., max. +9° C.		
D 20	3120	2380	23.7	3.11	99	—	H 2.36 M 1.33	89	+	—	4.76	90	+	16	Temperature during experiment: min. +3° C., max. +10° C.		
D 21	2900	2550	12.1	3.19	93	—	H 2.36 M 1.38	87	+	—	6.26	85	+	50	Temperature +37° C. Died after 36 hours of starvation.		
D 22	2800	2200	21.4	3.11	100	—	H 2.83 M 1.08	90	++	+	9.20	80	+++	25	Temperature during experiment: min. +25° C., max. +27° C.		
PS 14	500	400	20.0	2.80	101	—	H 2.65 M 1.49	83	++	+	3	Died of pneumonia 5 days after thyroidectomy.		
1526	1800?	1350	25.0?	2.58	100	—	H 2.88 M 1.04	82	++	+	5	Stock animal. Suffered from pneumonia.		
Means	2713†	2151†	20.6	2.97	98	...	H 2.71 M 1.15	86	7.04	88		

* These figures are omitted from the means, since there was no visible fatty change either in the kidneys or in the liver of rabbit D 11, or in the liver of rabbit D 16.

† The means of body weights do not include the figures for rabbits PS 14 and 1526.

FATTY CHANGE IN KIDNEYS



FIG. 1.—Rabbit D 6. (+++). Series II. Fat content of Henle zone, 3.77 per cent. $\times 112$.

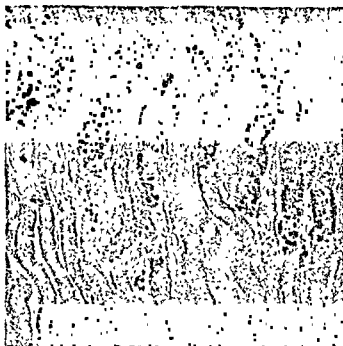


FIG. 2.—Rabbit D 12. (+++). Series II. Fat content of Henle zone, 3.41 per cent. $\times 112$.



FIG. 3.—Rabbit D 3. (++). Series I. Fat content of medulla (right kidney), 2.28 per cent. $\times 112$.



FIG. 4.—Rabbit D 13. Shows fatty change in kidney cortex (++). Fat content, 3.45 per cent. $\times 142$.

In figs. 1-3 the coarse fat globules are in the wide descending part of the Henle tubule; the fine granules are in the wide ascending limb.

This increase in fat in the Henle zone, which is out of all proportion to the increase in the other zones of the kidney, is particularly striking.

Histology. The intensity of the fatty change as seen in frozen sections stained with Scharlach R is indicated in the tables by conventional signs (\pm to $++++$) and is illustrated in figs. 1-4. The fat in all cases was isotropic. In only two animals (D 10 and D 13) did we detect any fatty change in the cortex and in the only animal (D 13) in which this change was well marked the highest

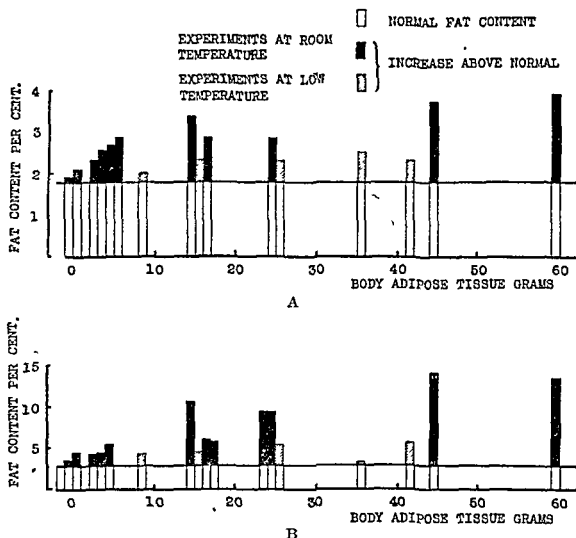


FIG. 5.—Relationship of fat content of (A) zone of Henle's tubules and (B) liver fat to bodily adiposity.

fat content was found. In the medulla the distribution of the fatty change was mainly in the broad portions of the ascending and descending limbs of Henle, and here the correlation between the estimation and the histological picture was well marked.

Correlation with fat reserves. The tables show the weight of the fat in the omentum and paravertebral regions. A general correlation between this and the quantity of fat appearing in the kidneys and in the liver is apparent (fig. 5). We have previously shown this to be a usual finding in organs in which fat mobilised from the depots accumulates in parenchymatous cells; such a relationship in the

kidney is therefore some evidence that the mechanism of the change is the same. This correlation, which is obvious in the earlier cases in table IV, is less obvious in animals D 14 to D 22, when they are considered with the other animals in this table. These, however, were all exposed to abnormal temperatures; this, as we shall show, causes considerable metabolic disturbance which must be taken into account.

One other point may be mentioned in this connection. Included in table IV are a number of animals which possessed only slight fat reserves at the conclusion of the experiments. As is to be expected, the degree of fatty change in the organs of most of these was minimal.

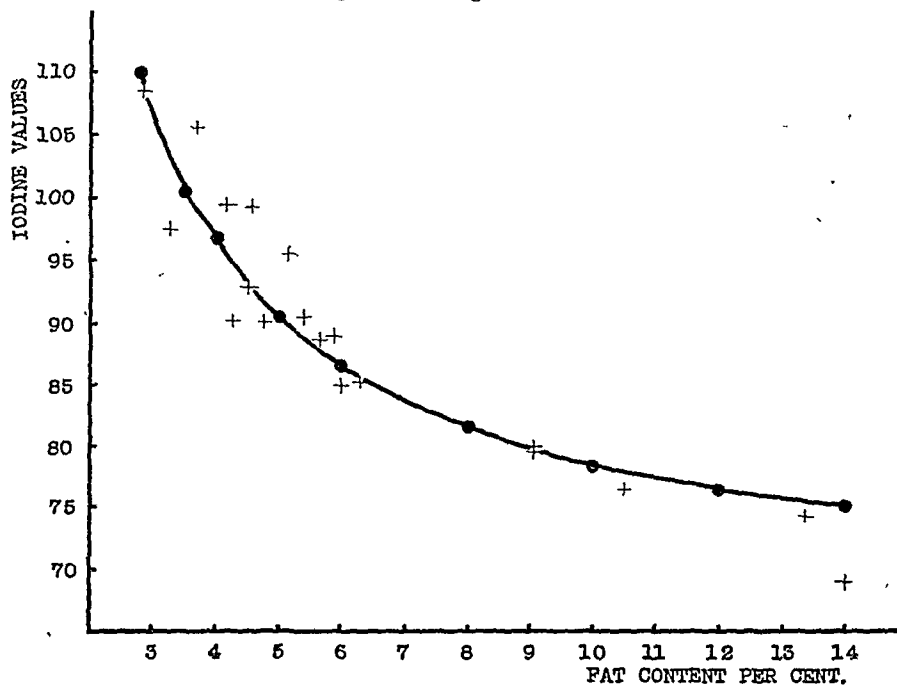


FIG. 6.—Liver fat content (per cent.) plotted against iodine values. Continuous curve calculated on the assumption that the increased fat content is due to infiltration by mobilised depot fat. + sign = actual findings.

and consequently the summarisation of the results as means, though convenient for presentation, fails to give a true picture of the extent of the change which can occur. This factor could have been largely eliminated if it had been possible to make sure that all the animals had adequate fat reserves before the stimulus of starvation was applied. Circumstances, however, prevented this.

Iodine values. These have been estimated in most cases. The iodine value of the depot fat of eighteen rabbits has been estimated and found to vary rather widely between 58 and 76, with a mean of 66.1 ± 7.2 . Since the iodine value of the combined (masked) fat

of the kidney is considerably higher (cortex, mean value about 103, medulla and Henle zone, about 95), it may be expected that an infiltration of depot fat should produce a significant lowering of the figure for the fat in the affected cells. This is the case. Thus the Henle zone of animal D 6 contains about double the normal quantity of fat which has an iodine value of 75, this is approximately what would be expected if an equal amount of depot fat of an iodine value of 66 were added to the same quantity of cellular fat of an iodine value of 95. This line of reasoning has been often used as evidence that the fat in the cells comes from outside and it has been shown that in the liver and heart the theoretical and actual values for a certain percentage of fat closely correspond (Imrie, 1914 15, 1922, Dible, 1934, 1938 39). The actual and theoretical relationships in the liver in our two series are shown in fig 6, and in the Henle zone of the kidney, in the experiments we are now considering, in fig 7. This sort of illustration of the change in the iodine value of cell fat by infiltration of fat of a lower value from outside is apt to be less striking in animals such as rabbits than in parallel observations in man, for the following reasons. Firstly, in man there is usually a greater difference between the iodine value of the essential (*élément constant*) fat and that of the depot fat than there is in these animals, and secondly, in the latter there is wider variation in the iodine value of the depot fat, due presumably to wider variations in diet.

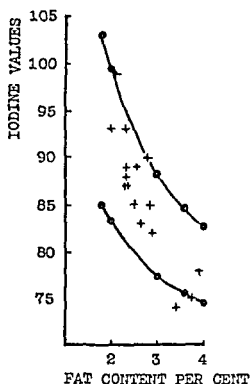


FIG 7 — Kidney fat content of Henle zone plotted against iodine values (+ signs). The two theoretical curves based upon the highest (103) and lowest (85) normal figures found for this zone are drawn on the assumption that any increase is due to infiltration with mobilised depot fat (I V 66).

PART II

THE INFLUENCE OF TEMPERATURE UPON FAT MOBILISATION IN STARVATION

We have already commented upon the fact that in the course of these experiments a number of observations were made which did not fit into the general trend of the results and those of previous experiments of this type. It is customary for the fatty change to

be developed best in animals which have abundant reserves of depot fat and there is, as a rule, a rough mathematical correlation between the quantities infiltrating the organs and those present in the depots. Reference to fig. 5 and table IV will show that there are a number of animals, *e.g.* D 14 and 15, which, whilst possessing good fat reserves, failed to show the expected change in the kidneys or liver to any but a slight degree. In casting about for an explanation of this phenomenon it was noticed that the experiments in question had been carried out at a time in winter when the windows of the laboratory had been broken by German bombs, and in consequence the temperature had fallen to unusually low levels. This possibility was explored and the other animals in the latter part of table IV (D 16 to D 22) were expressly used to investigate the effect of temperature.

It has been shown by Dible (1932) and Dible and Libman (1934) that the degree of fatty change which develops in experimental animals during starvation depends on the quantity of fat reserves the animals possess. In our experiments, therefore, the animals in which the effect of temperature on fat mobilisation can be compared are those which possess approximately the same amount of depot fat. Thus, it is justifiable to leave out of this kind of comparison at least animals D 2, D 9 and D 11 (see tables II and IV). The analyses of our results regarding the effect of temperature on fat mobilisation are presented in tables V and VI. We include

TABLE V

Animals starved at moderate or raised temperature

Animals	Kidney : Henle zone fat content (percentage of moist weight)	Liver fat content (percentage of moist weight)	Body adipose tissue (g.)	Temperature during experiments
D 3 (series I)	...	9.23	25.0	Room temp.
D 4 "	...	5.84	17.0	" "
D 5 "	...	5.40	5.0	" "
D 6 (series II)	3.77	14.01	45.0	" "
D 7 "	2.59	4.44	4.0	" "
D 8 "	2.85	6.01	17.0	" "
D 10 "	2.35	4.17	3.0	" "
D 12 "	3.41	10.56	15.0	" "
D 13 "	3.94	13.35	60.0	" "
D 22 "	2.83	9.20	25.0	25°-27° C.
Means	3.11	8.22	21.6	...

among the animals which were starved at room temperature D 5, D 7 and D 10, although they had less fat reserves than any of the rabbits starved at lower temperatures.

It is clear from these results that a low external temperature exercises a marked influence upon the quantity of fat accumulating in the parenchymatous tissues under the conditions of our experiments. Ohta (1940) in a recent paper reported similar results with rats.

TABLE VI
Animals starved at low temperatures

Animals	Kidney Henle zone fat content (percentage of moist weight)	Liver fat content (percentage of moist weight)	Body adipose tissue (g)	Temperature during experiments
D 14 (series II)	2.31	5.65	42.0	4°-10° C.
D 15 "	2.31	5.17	26.0	4°-10° C.
D 16 "	2.53	3.25	36.0	0°-10° C.
D 17 "	2.03	4.27	9.0	0°-9° C.
D 20 "	2.36	4.76	16.0	3°-10° C.
Means	2.31	4.62	25.8	.

The comparative mean figures for the relevant tissues in the animals starved at room temperature and in the cold are as follows.

	Henle zone fat content (percentage of moist weight)	Liver fat content (percentage of moist weight)	Body adipose tissue (g)
Room temperature	3.11	8.22	21.6
Cold	2.31	4.62	25.8

This difference is emphasised by the fact that, on the whole, the animals kept in the cold had slightly larger fat depots than those starved at room temperature. It should be noted that the animals were in open wire cages and wore wooden collars to prevent them eating their faeces. These collars and the confined space limited movements and they sat hunched up and relatively motionless. The effect on the mobilised fat is what might have been anticipated: the animals at a low temperature consume more fat to provide bodily heat and consequently the accumulations in the parenchymatous organs are diminished.

This assumption—that there is an increased consumption of mobilised neutral fat in animals kept in the cold—was tested by observing if there was any disturbance of the neutral fat/cholesterol ratio in the blood. Increased fat mobilisation in starvation leads to an increase in the blood fat and an accumulation in certain parenchymatous organs, since all of the fat mobilised is not immediately utilised: it was thought that, if there was excessive

utilisation of the neutral fat element for consumption, the ratio of this to the cholesterol should decrease.

The blood fats were estimated daily by Bloor's chromate oxidation method (Peters and Van Slyke, 1932). The quantity of blood taken for such estimation was 3.0 c.c. Control experiments showed that the removal of this quantity of blood at daily intervals does not affect the level of the blood lipoids. The total blood cholesterol was estimated from the same alcohol-ether filtrate which was used for the fat estimation; the amount used corresponded to 0.6 c.c. of whole blood.

As will be seen from tables VII and VIII, the neutral fat/cholesterol ratio markedly decreased during fasting at low temperature (from 3.22 to 1.30 and from 2.14 to 1.69). This decrease is especially

TABLE VII

Rabbit D 17. Blood lipoids as mg./100 c.c. during starvation at a low temperature: min. 0° C., max. 9° C. (mostly between 0° and 4° C.)

Date	Total fats	Cholesterol	Blood fats less cholesterol	Neutral fat/cholesterol ratio
Commencement				
7.1.41	287	68	219	3.22
8.1.41	269	69	200	2.9
9.1.41	281	104	177	1.7
10.1.41	269	85	184	2.16
11.1.41	275	111	164	1.48
13.1.41	258	112	146	1.3

TABLE VIII

Rabbit D 20. Blood lipoids as mg./100 c.c. during starvation at a low temperature: min. 3° C., max. 10° C. (mostly between 5° and 8° C.)

Date	Total fats	Cholesterol	Blood fats less cholesterol	Neutral fat/cholesterol ratio
Commencement				
5.2.41	264	84	180	2.14
6.2.41	318	100	218	2.18
7.2.41	317	105	212	2.02
8.2.41	342	110	232	2.11
9.2.41	326	118	208	1.75
10.2.41	338	123	215	1.75
11.2.41	350	130	220	1.69

striking in rabbit D 17, which was starved at a temperature very close to freezing point. In this rabbit, although there was an increase of blood cholesterol, indicating that fat mobilisation actually took place, the neutral fats have fallen to a low level. This, as we have already said, we believe to mean an excessive utilisation by the body to supply the higher metabolic requirements

at the lower temperature. Correlated with this, less fat is available for deposition in the parenchyma of the organs. It should be noted that histologically there was practically no fatty change in the liver of this rabbit.

We have also carried out a few observations on animals which were starved in an unusually warm environment (tables IX and X).

TABLE IX

Blood lipoids as mg/100 c.c. during starvation at a temperature of 37° C

Date	Total fats	Cholesterol	Blood fats less cholesterol	Neutral fat/cholesterol ratio
Rabbit D 18				
Commencement				
7.1.41	264	60	204	3.40
8.1.41	287	60	227	3.78
9.1.41	363	85	278	3.27
10.1.41	351	85	266	3.13
Rabbit D 21				
Commencement				
6.2.41	346	98	248	2.25
7.2.41	516	116	400	3.45

TABLE X

Rabbit D 22 Blood lipoids as mg/100 c.c. during starvation at a temperature of 25-27° C

Date	Total fats	Cholesterol	Blood fats less cholesterol	Neutral fat/cholesterol ratio
Commencement				
11.2.41	307	92	215	2.34
12.2.41	307	92	215	2.34
13.2.41	336	105	231	2.19
14.2.41	336	100	236	2.36
15.2.41	399	100	299	2.99
17.2.41	440	106	334	3.15

Two animals (D 18 and D 21) kept at 37° C succumbed in 4 days and 36 hours respectively. In these and in animal D 22, which was maintained at 25-27° C, fat mobilisation occurred, but the neutral fat/cholesterol ratios showed a tendency to rise. The extent of the fatty infiltration in the organs of these animals was similar to those kept at room temperature.

SUMMARY AND CONCLUSIONS

In the fatty change which is induced in the rabbit's kidneys by starvation the greatest concentration of fat occurs in the Henle zone: this can be shown by dissection and chemical analysis to be a real increase which quantitatively accords with the histological appearances.

The evidence of increase in quantity, reduction in iodine value and relationship of the increase in fat to the extent of the animal's storage depots indicates that the change is an infiltration.

At low temperatures the usual quantitative relationship between depot fat and the degree of fatty infiltration in the parenchymatous organs is upset, owing to the greater utilisation of the mobilised fat to meet the animal's increased calorific requirements.

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SHORT ARTICLES

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HETEROTOPIC BONE MARROW IN THE RENAL HILUM CAUSING PYONEPHROSIS IN AN ADULT WITH CHRONIC MYELOID LEUKÆMIA

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(PLATE VIII)

Massive deposits of extra-medullary bone marrow are rarely observed in the adult. Small, usually microscopical islands of hæmopoietic tissue, however, have been described in many different organs in certain cases of disordered hæmopoiesis. The condition is more frequent in infants than in adults, especially infants suffering from anæmic conditions, as in *icterus gravis neonatorum* (Hawksley and Lightwood, 1934; Parsons, 1938). Brannan (1927), in an exhaustive survey of the literature, noted that these deposits are frequently found in cases presenting Von Jaksch's syndrome. He collected ten cases from the literature where the deposits formed tumour-like masses in the renal hilum and described a further case. No patient was more than two years old. In the case described by Parkes Weber (1918-19), the patient was a child with acute myeloid leukæmia and severe anæmia. In the adult, macroscopic deposits of extra-medullary bone marrow have been described in the thorax in acholuric jaundice and allied conditions. This aspect of the subject is reviewed by Hartfall and Stewart (1933). Deposits of true extra-medullary bone marrow do not appear to have been described in chronic myeloid leukæmia. Furthermore although in this disease the kidneys are frequently the seat of leukæmic infiltration, diffuse or nodular, urinary symptoms are uncommon (Forkner, 1938).

Case history

The patient, a male aged 73 years, was admitted to hospital following a fall. He stated that he had been quite well until nine months previously when, following an attack of "influenza", he was seized with abdominal pain and noticed a "lump" in his abdomen. Simultaneously he developed excessive thirst, drinking 10-12 pints of fluid a day.

On examination there was bruising around both eyes. The teeth had been removed and the mouth and pharynx were healthy. No glands were palpable in neck, groins or axillæ. There was a soft systolic murmur in the mitral area. Blood pressure was 130/60. The chest was barrel-shaped. The central nervous system was healthy.

Abdominal examination showed the liver to be enlarged four fingers-breadth below the costal margin. The left loin was occupied by a large smooth hard mass, which presented no notch: it moved freely with

respiration. The urine had a specific gravity of 1008 and showed a trace of albumin. Later it was found that the average daily excretion of urine, measured over 28 days, was 76 ounces.

A tentative diagnosis of renal tumour was made. Excretion pyelography failed to reveal the kidney shadows. Cystoscopy was performed by Mr D. N. Matthews. Pus was seen in the bladder but retrograde pyelography could not be performed owing to hæmorrhage on catheterising the left ureter.

At this point a blood examination revealed the presence of a chronic myeloid leukaemia.

TABLE
Blood counts

Date	4.10.40	17.10.40	24.10.40	25.10.40
Hæmoglobin . . .	92 per cent. (Haldane)	90 per cent.
Red blood cells . . .	5,050,000	5,260,000	5,590,000	5,870,000
Colour index . . .	0.9	0.8
White blood cells . . .	54,500	38,400	45,800	45,600
Polymorphs . . .	19,075 (35 per cent.)	18,240 (40 per cent.)
Eosinophils . . .	1635 (3 ")	13,224 (29 ")
Basophils . . .	0	2736 (6 ")
Neutrophil myelocytes	31,610 (58 ")	4104 (9 ")
Myeloblasts . . .	545 (1 ")	0
Lymphocytes . . .	1635 (3 ")	2736 (6 ")
Monocytes . . .	0	4560 (10 ")
Nucleated R.B.C. . .	Many seen	912 per c.mm. (normoblasts)

Stained films showed some polychromasia and a great increase in the number of platelets. There was a slight increase in the total number of red cells during the three weeks that he was under observation (he was never anæmic), while a notable increase in the number of eosinophils and basophils coincided with a great reduction in myelocytes and a complete disappearance of myeloblasts. These changes are probably the result of one application of X-rays on 11.10.40. The blood urea on 1.10.40 was 76 mg. per 100 c.c.

The patient received two applications of deep X-rays to the spleen, 1200 r to an anterior field on 11.10.40 and 1500 r to a lateral field on 30.10.40. His general condition, however, deteriorated rapidly, he developed thrombotic ulcers in the natal cleft, on the inner side of the right cheek and on the outer side of the right arm. He died of bronchopneumonia on 6.11.40, 49 days after admission.

Post-mortem findings

There were necrotic ulcers of the left cheek, the natal cleft and the outer side of the left arm. Tracheo-bronchitis and bilateral bronchopneumonia were present. The heart muscle showed fatty degeneration, while the valves showed some calcification. The œsophagus and stomach were healthy. The small and large intestines showed congestion only. The lymphatic glands throughout the body were not enlarged. The liver was enlarged, weighing 96 oz. On section it was pale, with a finely granular cut surface. The spleen weighed 73 oz. and was covered by the firmly adherent omentum. On section it was greyish-pink in colour and very firm. The capsule was much thickened and rough on its outer surface. There was leukæmic infiltration between the dura mater and the arachnoid over the vertex of the brain, which itself showed no naked eye involvement. The pituitary

BONE MARROW HETEROPTOPIA



FIG. 1—Left kidney in vertical section showing dark red mass of heterotopic bone marrow replacing the fat in the hilum $\times 4$ approx.



FIG. 2—Right kidney in vertical section showing grey mass of heterotopic bone marrow in the hilum, obliteration of pelvis and calyces and an abscess cavity near the upper pole \times approx.



FIG. 3—Section from hilum of right kidney showing relation of heterotopic marrow to pelvis. The giant cells are megakaryocytes H and E $\times 100$.



FIG. 4—Nest of immature red blood cells (erythroblasts and normoblasts) from hilum of left kidney. Leishman's stain $\times 900$.

gland did not appear to be diseased. The left kidney (fig 1) was 5 inches long and weighed 10 oz. A dark purple mass occupied the hilum, replacing the pelvic fat and surrounding the pelvis and calyces. The right kidney (fig 2) showed a somewhat similar but grey mass of tissue. There was a pyonephrosis at the upper pole, containing very foul smelling pus. This kidney was 4½ inches long and weighed 13 oz. Both ureters were patent. The urinary bladder showed cystitis and contained purulent material similar to that found in the right kidney. The prostate was not enlarged.

The bone marrow of the ribs and sternum only was examined. It was diffusely hyperplastic.

Histology

Sections of the liver, spleen and dura mater show typical infiltration with immature granular leucocytes. No foci of hæmopoiesis are seen in the portions examined.

The parenchyma of the *right kidney* shows inflammatory changes suggesting pyelonephritis. There is considerable renal fibrosis and many glomeruli are obliterated. The mass in the hilum is bone marrow lying external to the renal pelvis (fig 3).

The parenchyma of the *left kidney* shows a few scattered accumulations of immature granular cells; otherwise it is essentially healthy. The mass in the hilum is again bone marrow (fig 4). These masses in each hilum (figs 3 and 4) are found to be much congested, the capillaries being dilated and containing chiefly white blood cells. The stroma is packed with aggregates of immature leucocytes—myelocytes and myeloblasts—together with foci of nucleated red blood cells in all stages of development (fig 4). Many megakaryocytes are seen scattered throughout the tissue and foci of adipose tissue are also present. No free iron is detected. It will be noted that the heterotopic tissue does not invade the renal parenchyma, from which it is separated by a zone of fibrous tissue. The appearances contrast sharply with those of leukaemic infiltration of the renal parenchyma as commonly encountered.

Comment

The heterotopic foci of bone marrow found in infancy are presumably relics of the multicentric hæmopoiesis of foetal life. In the case described it would appear that such foci, dormant for 73 years, were reactivated by the onset of the leukaemic process. The pressure of the hilar mass on the right side was probably responsible for the pyonephrosis.

This case differs from most of the others described in the literature in that the patient was not anæmic, showing that the hæmopoietic marrow, both intra and extra medullary, was fully capable of responding to the strain put upon it.

None of 26 cases of leukaemia found in a series of 3720 consecutive post mortems at the Westminster Hospital showed any renal lesion comparable to that found in the present case.

Summary

Bilateral tumour like masses of true bone marrow occurring in the renal hilum are described in a male aged 73 years with chronic myeloid leukaemia, pyonephrosis had resulted in one kidney. The appearances contrast strikingly with leukaemic infiltration of the renal parenchyma as commonly encountered.

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OBSERVATIONS ON THE BIOCHEMICAL ACTIVITY OF
PSEUDOMONAS PYOCYANEA

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Statements regarding indole production and fermentation of carbohydrates by *Pseudomonas pyocyanea* are somewhat divergent. Wilson (1929) states that indole is either not produced or is formed only in small amounts, Topley and Wilson (1936) that the indole test is usually negative but may be positive and Bergey (1934) that indole production differentiates *Ps. pyocyanea* from *Ps. fluorescens*. Wilson states that acid is produced from glucose, as do Topley and Wilson, but Bergey (1934) says that none of the carbohydrates commonly used are fermented. Sandiford (1937) found that none of his 50 strains produced indole but that they fermented glucose without production of gas and this view is partly adopted by Bergey (1939).

In this paper are recorded the results of a study of 85 strains isolated from urine, fæces and various body fluids and discharges. All the strains were motile, produced ammonia and peptonised milk, and all were frank producers of the greenish-blue pigment pyocyanin.

Ps. pyocyanea has been isolated from blood, urine, fæces and various inflammatory conditions, either alone or in combination with other organisms. The great majority of the positive urines were from females. Exposed wounds and superficial ulcers often harbour this organism. Whether or not it can alone cause acute diarrhoea in infants is uncertain, but it has been present in a high proportion of fæces examined from this source. It is not so frequently found in throat swabs, but its more common occurrence, often alone, in pus from otitis media and mastoiditis would seem to indicate that it has a predilection for these sites. One of the blood strains was from a suspected streptococcal septicæmia secondary to panophthalmitis. Repeated blood culture from this patient yielded no other organism and swabs from the eye yielded nothing else that might have accounted for the fatal

septicæmia. Another positive blood culture was obtained in a case of lung abscess. Two specimens of blood from patients suspected to be suffering from enteric fever yielded both *Ps pyocyanea* and *Bact typhosum*. It is possible that the 18 positive blood cultures, 9 of them pure cultures, may have been due to contamination. While the organism is usually of low virulence, it would appear that one may occasionally encounter a more virulent strain which may even cause death.

The culture media employed were Dunham's peptone water (pH 7.2) made with Difco peptone, one per cent sugar solutions in the same medium with Andrade's indicator and Koser's citrate solution. Most of the experiments were made with 72 hour cultures at 37° C but, when a high concentration of pigment was desired, the tubes were kept at laboratory temperature (25°-30° C) for one day more. When necessary, cultures were studied at different ages up to ten days. The tests for indole were those of Holman and Gonzales, as quoted by Topley and Wilson, and of Goré (1920-21). As pointed out by Sandiford the addition of the Bohme reagents—potassium persulphate and paradimethylamidobenzaldehyde—separately to a culture of *Ps pyocyanea* produces a pink colour, the intensity of which in both cases varies with the intensity of the green colour of the test cultures. The same alteration is produced by the addition of a drop of dilute HCl to a fresh culture and the original colour is restored by the addition of a drop of strong alkali. No such pink colour is produced by the addition of persulphate solution or acid to a culture of *Bact coli* containing indole or to a solution of indole in peptone water.

To see whether indole was really present, its reaction being masked by the alteration in colour of pyocyanin on the addition of the Bohme reagents, cultures were shaken separately with ether, xylene, benzene or ligroin, in which indole is soluble but not pyocyanin. The separated solutions gave no indole reaction with the Bohme method but the residue of the culture after extraction gave the pink colour with persulphate solution alone. The Gore method also invariably gave negative results. According to Salle (1939) the oxalic acid paper test is specific for indole, as this is the only volatile compound formed by microorganisms which is capable of producing a pink colour with oxalic acid crystals. None of the strains of this series reacted positively to this test. These experiments seem to prove conclusively that *Ps pyocyanea* does not form indole in peptone water.

Ps pyocyanea grows well in Koser's citrate solution but without the production of pigment. The addition of lactose, maltose or glycerol encourages the synthesis of pyocyanin. On the addition of either of the Bohme reagents or of acid to such pigmented cultures, the same change from greenish blue to pink was seen, due to the effect of acid on pyocyanin. Indole could not have been present and so was not necessary for the change of colour to occur.

The change from greenish blue to pink in an acid medium is probably due to the formation of an acid compound of the pigment which is pink in watery solution. This coloured salt is soluble in phenol but not in chloroform, whereas pyocyanin is soluble in chloroform as well as in phenol. Pyocyanin seems to be a delicate indicator. It is not affected by boiling for fifteen minutes.

Ten strains were grown in Koser's citrate solution (pH 7.0) at 37° C. The reaction in all became alkaline to litmus in 24 hours and at 72 hours the pH of the different cultures ranged between 7.6 and 8. Moreover peptone water cultures, after extraction of the pigment with chloroform, showed a high degree of alkalinity, often above pH 8. The capacity of the individual strains to form alkali under these conditions varied.

All the strains were inoculated into peptone water containing one per cent. of various sugars and Andrade's indicator. The cultures were incubated at 37° C. and observed for at least 14 days. It was invariably found that glucose and galactose were attacked in 24-48 hours with the production of acid but not gas. None of the other sugars showed any acid change. In all media except those containing glucose and galactose green pigment was formed to a greater or less extent.

Solutions of the various sugars employed above were made in peptone water without the addition of indicator and *Ps. pyocyanea* was grown in these under similar conditions. Only those containing glucose and galactose showed any pink colour although there was pigment production in all. The glucose tubes were more pink than the galactose and a drop of alkali added to either caused the disappearance of the pink colour and the appearance of the greenish-blue hue. This clearly shows that the pink colour in the glucose and galactose tubes was due to a change in the colour of pyocyanin caused by the acid produced during growth. Other cultures were made in Koser's citrate solution containing glucose and indicator. No pigment was formed but an acid reaction developed, thus proving that the organism is able to break down glucose, producing sufficient acid to change the indicator irrespective of other cultural surroundings.

From these experiments the conclusion is drawn that *Ps. pyocyanea* can ferment glucose and galactose without gas production. It would appear that the amount of acid produced, in glucose at least, is not necessarily low but that a portion of it is diverted to neutralise the alkali formed by the organism during its growth and therefore the quantity available to affect the indicator is not great.

Summary

None of 85 strains of *Ps. pyocyanea* examined produced indole; all formed acid but not gas from glucose and galactose.

The fact that pyocyanin turns red in an acid medium possibly accounts for the positive indole reaction reported by some workers.

Ps. pyocyanea forms alkali during growth on media containing no fermentable carbohydrate.

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PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

4th July 1941

The sixty second meeting of the Society was held in the Department of Pathology, University of Glasgow, on Friday 4th July 1941

Communications and demonstrations

The items marked * are abstracted below

- IVY MACKENZIE Pituitary tumour and organic brain disease
- A LYALL and R D STUART. Spreading gangrene of the skin
- W. GILBERT MILLER. Aortic size and accidental death.
- RAYMOND WHITEHEAD The fat of the adrenal cortex in starved guinea-pigs and rabbits
- J. F. HEGGIE. The glomerular capillary circulation: (1) in the normal animal (rabbit), (2) in the development of renal cortical necrosis
- J. W. CLEGG. The diagnosis of Gaucher's disease by sternal marrow puncture
- L. G. L. BYWATERS and J. HENRY DIBLE The renal lesion in traumatic anuria.
- A C. LENDRUM. Anaemia with splenomegaly and a positive Wassermann reaction
- C. E. VAN ROOYEN, A J. RHODES and A CAMERON. Herpes labialis following sulphapyridine and T. A. B injection.
- L R. W. PRICE. An unusual case of toxæmia in a munition worker.
- C H. BROWNING and W. M. LECKIE. Depression of experimental streptococcus infection in mice by drugs of the sulphonamide group and the activating effect of *p* aminobenzoic acid (Woods and Selbie)—an example of therapeutic interference.
- C. H. BROWNING and K. M. CALVER Treatment of *T. congolense* infection in mice by a phenanthridinium compound (Morgan and Walls): cure after one or more relapses, each treated by the same single dose not greater than that used originally.
- R. D. PASSEY Experimental canine tar tumours.
- PAUL BROWNING Sarcoma produced by a new water soluble compound, 2 (3' methyl 4' hydroxyethylamino) styryl 6-*p* acetylaminobenzoyl-amino quinoline methoacetate.

P. R. PEACOCK and STEPHAN BECK. Avitaminosis-A and papillomatosis of the fore-stomach of rats, induced by feeding with heated fats.

J. W. BIGGER and J. H. NELSON. Growth of coliform bacilli in distilled water.

A. FLEMING. Some uses of nigrosin in bacteriology.

*LEONARD COLEBROOK and A. E. FRANCIS. Varying sensitivity of hæmolytic streptococci to the sulphonamides.

*D. McCLEAN. Further observations on the capsulation of streptococci and its relation to diffusion factor (hyaluronidase).

R. D. STUART. The value of liquoid in blood cultures.

H. J. PARISH and H. PROOM. Some observations on the diphtheria virulence test.

P. BROWNING. Sarcoma production in rats by styryl-430.

J. N. COOK, C. L. HEWETT, E. L. KENNAWAY and N. M. KENNAWAY. Liver tumours produced by 2 : 2'-azonaphthalene.

*M. GILLESPIE, J. S. F. NIVEN and J. SHAW DUNN. The renal lesion in cases of "crush" syndrome.

T. HEWER. (1) Primary cerebral vein thrombosis. (2) Two cases of renal vein thrombosis.

J. KIRKPATRICK. Flagellated forms of *Tr. pallidum* and other spirochætes from human sources.

J. KIRKPATRICK and A. C. LENDRUM. Demonstration of histological and bacteriological preparations mounted in synthetic (D.P.X.) medium.

A. C. LENDRUM. Chondroma of lung.

T. GLEN LISTON. Trichomonas infestations.

N. G. B. McLEITCH. (1) Hyalinisation of the basophile cells in the anterior pituitary. (2) Carcinoma of adreno-cortical rest.

J. S. F. NIVEN. (1) Blast lesion of lungs and early nephritis acris. (2) Malignant mixed tumour of nasopharynx.

L. R. W. PRICE. Four cases of sarcoma of the urinary bladder.

I. RANNIE and D. S. STEVENSON. Nesidioblastoma.

Abstracts

576.851.2 (streptococcus): 615.778

VARYING SENSITIVITY OF HÆMOLYTIC STREPTOCOCCI
TO THE SULPHONAMIDES

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By means of "ditch plates", *i.e.* blood agar plates of which a strip across the centre has been replaced by the same medium containing one of the sulphonamide compounds (Fleming's method, 1940 41), a few hæmolytic streptococcal strains have been met with which are more or less completely insensitive to these drugs. Most of these strains were isolated from wounds in which they had persisted in spite of powdering with sulphanilamide. In several instances their presence had been associated with a febrile disturbance following grafting operations—and the failure of such grafts.

The "ditch plate" method is very convenient but certain pitfalls must be recognised. (a) Some batches of agar antagonise sulphanilamide and cannot be employed. They can only be found at present by trial and error, using known sensitive and insensitive strains. (b) The inoculum must be small or there will be no inhibition. A 1:2000 dilution of an overnight serum broth culture will usually be appropriate. (c) The plates should be made 3–5 hours before use, kept in the refrigerator, then dried off and placed in the incubator immediately after planting. If this procedure is not adopted the following variations from the usual result may be obtained: (1) there may be no inhibition of growth (if the plate is not incubated at once), (2) there may be no growth—*i.e.* inhibition to the edge of the plate if the sulphonamide has had time to diffuse too far, (3) a curious phenomenon may be observed, namely growth of sensitive strains over the ditch and perhaps a little beyond, then a zone of inhibition, then normal uninhibited growth, the explanation of this is not clear.

For quantitative work, *i.e.* to detect possible changes of sensitivity during treatment of a patient by sulphonamide, the ditch plate is not likely to be so useful as incubation of small inocula of streptococci in heated human blood (47° C for half an hour to kill leucocytes) *plus* graded additions of drug and subsequent viable counts.

No survey has yet been made of all serological types and groups of hæmolytic streptococci. Resistant strains belonging to four types—12, 13, 25 and the provisional Griffith type B 3264—have been met with. Strains of groups C and G have usually been very sensitive.

Strains resistant to sulphanilamide have proved almost equally resistant to sulphapyridine, sulphathiazole and sulphadiazene, but further observations are necessary.

There is no definite evidence yet as to whether resistance to sulphanilamide has developed during treatment.

The recognition of these resistant strains raises several points of interest. (1) In considering what dosage is required it will be necessary to take into account not only the concentration of the drug in the blood and tissues, but also the degree of sensitivity of the infecting organism. (2) The presence of resistant strains in a surgical ward with many open wounds may well lead to cross infections, because of the persistence of these organisms. Out of 14

such cross infections occurring during a period of 5 months in one ward no less than 10 were due to transfer of a single resistant strain (type 12). Recent experience also suggests that the presence of these strains in a wound should always be excluded before the surgeon undertakes a plastic or grafting operation, since any ensuing sepsis will not be controllable by the sulphonamides. (3) It may explain the occasional failure to control an acute streptococcal infection by the sulphonamides. (4) It may facilitate study of the mode of action of these compounds.

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576 . 851 . 2 (streptococcus) : 576 . 809 . 485

FURTHER OBSERVATIONS ON THE CAPSULATION OF STREPTOCOCCI AND ITS RELATION TO DIFFUSION FACTOR (HYALURONIDASE)

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The statement recorded elsewhere in this journal (McClean, 1941) that "mucoid" group A colonies on plates fail to produce haemolysis requires modification in the light of further observations. It has been found that the absence of haemolysis was due to the use of sheep's blood and the inclusion of glucose in the medium. When horse blood is used a definite zone of haemolysis develops which is reduced in area by the presence of glucose in the medium. The fact remains that, in the presence of sheep's blood, "mucoid" or capsulated strains produce little or no haemolysis, whereas non-capsulated hyaluronidase-producing strains produce wide zones of haemolysis in this medium.

It has been reported that the viscosity of hyaluronic acid is reduced by several reducing substances (McClean and Hale, 1941). It was of interest, therefore, to determine whether the disappearance of the hyaluronic acid capsule of streptococci as the cultures age is associated with any change in the oxidising or reducing conditions in the culture medium. A number of oxidation-reduction dyes in a graded series were included in the medium and the rate of their decolourisation was noted and correlated with the pH of the medium and the state of capsulation of the streptococci. The maximum reduction of the dyes and fall in pH had occurred after 4 hours' incubation, when the streptococci were still fully capsulated. The capsules disappeared between 6 and 24 hours, during which period no further change in the reduction of the dyes or pH occurred. It appears, therefore, that the decapsulation of streptococci is not directly associated with any increased reducing activity of the culture brought about by the metabolism of the organisms.

Daily serial subcultures of hyaluronidase-producing strains of streptococci in medium containing hyaluronic acid have indicated that there is no further increase in the production of hyaluronidase after the first subculture in this medium. Furthermore, when these passaged strains are returned to an

ordinary medium the production of this enzyme falls to its ordinary level. Capsulated strains serially cultivated in the same medium do not develop the power to produce hyaluronidase. Capsulated strains serially cultivated in medium containing hyaluronidase fail to develop capsules while in this medium, but show capsules again immediately on their return to an ordinary medium. These observations indicate that those strains that can secrete this enzyme make an immediate maximum response to the presence of the appropriate substrate. Similarly the capacity of capsulated strains to synthesise hyaluronic acid is only temporarily inhibited by the presence of hyaluronidase.

Twenty two strains of group G streptococci have been examined. Only four of these strains develop capsules and sixteen produce hyaluronidase. The capsules of one of these strains are composed of hyaluronic acid being destroyed by hyaluronidase, and free hyaluronic acid is found in older cultures of this strain when the capsules have disappeared. The capsules of the other three strains are not composed of hyaluronic acid nor is this material found in the medium. Only two group L strains have so far been examined, neither develop capsules and both produce hyaluronidase to a moderate titre. Only one group M strain has been available so far, this strain develops well marked capsules which are not composed of hyaluronic acid and, moreover, the capsules of this strain persist up to 20 hours in serum glucose broth. It is hoped to examine more representative strains of these and other groups. I am indebted to Dr R. M. Fry of the Emergency Public Health Service, Carmarthen, for the supply of strains.

Preliminary infection experiments in mice and rabbits have yielded some interesting results. Following intraperitoneal injection in mice, it is found that the uncapsulated hyaluronidase producing strains are of very low virulence, but that the capsulated group A and C strains show very great variation in the virulence of different strains, some of these strains are as avirulent as those that produce hyaluronidase, which suggests that virulence is not determined solely by the capacity to develop a capsule. When mice are infected by the intracutaneous route the relative lethality of some of the hyaluronidase producing strains compared with capsulated strains is greatly increased. It has been difficult so far to draw any definite distinctions between the types of local lesion produced by these strains in mice. If, however, intracutaneous injections are made in rabbits it appears that the capsulated strains produce a lesion which is definitely circumscribed and may either resemble a furuncle in appearance or develop the character of a raised indurated area with or without some central necrosis. The hyaluronidase producing strains on the other hand, cause a large area of necrosis, tracking in the direction of gravity and surrounded by an indurated erythematous area without well defined margins. A comparative study of the types of lesion produced in the skin by a large number of strains is required and it is hoped to investigate the influence of anti hyaluronidase serum, but the preparation of this serum depends upon the successful purification of the enzyme and its separation from streptococcal hemolysin.

The influence of hyaluronidase on the virulence and *in vivo* capsulation of streptococci has been examined. In some experiments the dilution of culture and a volume of potent enzyme have been mixed and injected together into the peritoneum, the capsules of the streptococci were shown to have been destroyed by the enzyme before injection. In other experiments 0.5 ml. of a solution of hyaluronidase known to destroy the clotting power of mucin in a dilution of 1:1000 was injected intravenously before and at intervals after, intraperitoneal injection of dilutions of a virulent capsulated strain. Notwithstanding the fact that the concentration of hyaluronidase present

in the circulation must have been much greater than that required to produce in-vitro decapsulation of the streptococci, the enzyme exerted no influence on the number of mice that died in these groups or on the time of death compared with the control groups that had received culture alone. Moreover, the streptococci in the peritoneum and heart blood were fully capsulated. Thus it appears that there is some in-vivo inhibition of the action of hyaluronidase on streptococcal capsules; this is in agreement with the observation that, notwithstanding the enormous doses of enzyme injected, the mice show no signs of blindness or embarrassed movement such as might have been expected if the vitreous humour or synovial fluid had been hydrolysed. The cause of this apparent inhibition of enzyme activity is being examined.

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THE RENAL LESION IN CASES OF
 "CRUSH" SYNDROME

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Case 1. V. W., female aged 18, sustained severe injuries which involved crushing of the legs and pelvis. Oliguria developed and the blood urea rose steadily to 440 mg. per cent. at the time of death five days later. *Post mortem* there was oedema of the legs and vulva; an area of necrosis 6 cm. in length was found in the right quadriceps femoris. The kidneys were enlarged and the cortex pale but streaked with red and yellow. An opaque whitish zone 1.2 mm. broad was noted at the bases of the medullary pyramids; the latter were dark and congested. On microscopic examination the glomeruli and first convoluted tubules appeared fairly normal apart from the presence of albuminous debris in the form of granules or light spheroidal masses in Bowman's capsules and the tubular lumina. The second convoluted tubules in the cortex mostly showed some evidence of recent regenerative change with increase of nuclei. Many of them contained brownish cast material in the form of clumps or ribbons of beads, or in amorphous masses. The most notable changes occurred in the medullary rays and in the outer medullary stripe, where there was frequent evidence of recent severe damage of ascending limbs of Henle's loops, followed by active regeneration of tubular epithelium and by proliferation of interstitial tissue. In serial sections it was ascertained that there were numerous foci of maximal damage at points where the affected tubules passed close to the walls of venules. At each of these points the tubule was dilated in an aneurysm-like bulge and the lumen was filled with an eosinophile coagulum. In many instances the bulging tubule had ruptured into the venule and thrombus had formed at this point. These lesions, which were very numerous, were all in a healing

state, having probably originated acutely soon after the injury. In the medulla, brown casts as described by Bywaters and Beall (1941) were present in many collecting tubules.

Case 2. In a second case of the same kind the kidneys had been the seat of antecedent changes due to arteriosclerosis and the more recent lesions were difficult to decipher. The same lesions as those described above could be recognised, however, including a few of the tubulo-venous foci.

The oliguria, low urea concentration and urea retention in these cases are features commonly observed after acute damage of tubules, as in experimental nephritis, irrespective of whether upper or lower segments of the nephrons are affected. In the present cases the lower segments appear to be almost exclusively involved: this localisation of lesion has been obtained experimentally by dosage with uric acid (Dunn and Polson, 1926) and with phosphate (McFarlane, 1941).

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CHRONIC RENAL DISEASE, SECONDARY PARATHYROID HYPERPLASIA, DECALCIFICATION OF BONE AND METASTATIC CALCIFICATION

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(PLATES IX XI)

THE syndrome which forms the subject of this paper is a rare one and consideration of the chemical and physiological mechanisms involved in it raises many interesting questions. We give here a report of a case showing all the characteristic features of the syndrome, together with a survey of similar cases in the literature and a discussion of the various factors involved.

CASE REPORT

Clinical history

The patient was a married woman, aged 40, who complained of ill health since the birth of a child four years before. During the latter part of this, her only pregnancy, she had suffered from hæmaturia, swelling of the face, backache and vomiting, diagnosed as acute nephritis. The child was six weeks premature and died at birth. After the birth of the child the hæmaturia ceased, but the patient continued in ill health and suffered from increasing weakness, polyuria, nocturnal frequency of micturition and intermittent vomiting. For a year before her admission to hospital she suffered from pain in the region of joints, worse at night and affecting at different times the knees, ankles, wrists, elbows, shoulders and spine. There was no swelling of the joints. During the same period the ends of the fingers became swollen, painful and tender. For six months before admission there were also severe headaches, breathlessness, incapacity for exertion, swelling of the ankles and feet in the evening and increasing pallor of the skin. There had been nothing of significance in the family or personal history prior to this four years' illness.

Examination showed a weary looking woman with extreme pallor and a slight icteric tinge. The finger ends were bulbous and cyanosed but the nails flattened and striated, rather than clubbed, the pulp of the right middle

finger was red and very tender, with two yellow subcutaneous nodules deeply situated. Above the left acromio-clavicular joint was a soft, lobulated, fluctuant swelling the size of a walnut. The radial artery was remarkably rigid and thickened—a typical “pipe-stem” artery. All palpable parts of the arterial tree showed similar changes in greater or less degree. The apex beat was diffuse, but maximal in the fifth interspace within the nipple line, and there was a marked systolic thrill at the apex accompanied by a loud rasping murmur audible all over the front and back of the chest. The blood pressure was 180/110 mm. Hg. The chest and abdomen showed no abnormal physical signs. There was no abnormality in the central nervous system or optic fundi.

Laboratory data

Urine. The specific gravity was 1016 in each of six four-hourly specimens. There was heavy albuminuria and the deposit showed occasional red cells, leucocytes and epithelial cells, but no casts. *Blood analyses:* urea 300 mg. per 100 c.c., calcium 11.7 mg. per 100 c.c. serum, inorganic phosphate 7.8 mg. P per 100 c.c. plasma, phosphatase 32.4 units (Jenner and Kay's method; normal 3.2 to 7.9). *Wassermann reaction* negative. *Blood cultures* sterile. *Blood count:* red cells 1,940,000 per c.mm., hæmoglobin 38 per cent., colour index 0.98, total leucocytes 7400 per c.mm., differential leucocyte count normal.

Radiological investigation

Seven months before admission, a radiograph of the right knee joint showed deposition of opaque material in or near the insertion of the patellar tendon and calcification of the surrounding arteries. On admission (Feb. 1940) a radiograph of the chest showed symmetrical destructive lesions of the acromio-clavicular joints, while projecting above the left was an ovoid mass of equal density to the bones (fig. 1). Striking changes were seen in the hands. Smooth, rounded clusters of opaque material were deposited at the tips of the thumb and all fingers in the left hand (fig. 2) and all save the ring finger in the right hand. There appeared to be absorption of portions of some of the terminal phalanges. Similar deposits were seen in the soft tissues of the left middle finger and between the radial styloid and the first metacarpal bone. Marked calcification was evident in the interosseous arteries. Direct views of the abdomen showed extreme calcification of the splenic and iliac arteries. No urinary calculi were seen. There was no dye excretion after intravenous uroselectan.

The clinical diagnosis, made on consideration of these findings, was chronic glomerulo-nephritis and general metastatic calcification, in which the mitral valve was probably involved. In view of the widespread calcification and evidence of chronic renal disease, the possibility of secondary hyperplasia of the parathyroid glands was suggested.

Clinical course

The patient remained in hospital for a month. Her condition deteriorated and clinical uræmia became increasingly evident. She returned home and died two days later.

Post-mortem examination

The autopsy was performed in the patient's home and was therefore limited in scope. The central nervous system was not

METASTATIC CALCIFICATION



FIG 1—Radiograph of chest showing destructive lesions of the acromioclavicular joints and opaque material above the left acromioclavicular joint

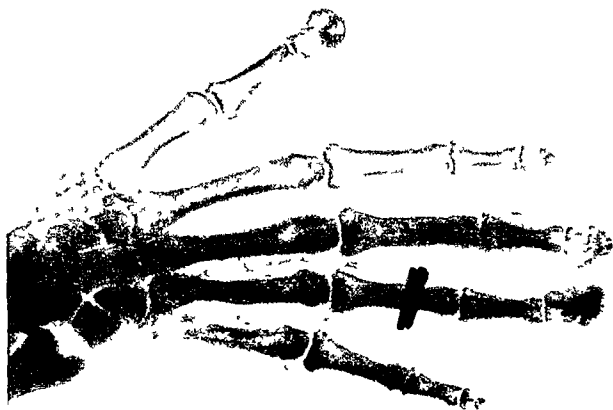


FIG 2—Radiograph of left hand showing deposition of opaque material around terminal phalanges and calcification of interosseous arteries

METASTATIC CALCIFICATION

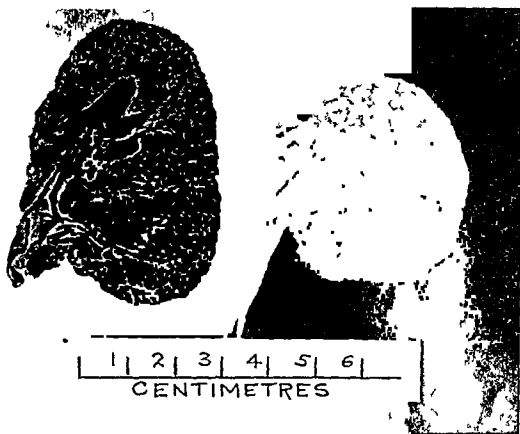


FIG. 3—Right and left kidneys showing marked reduction in size granular external surface and loss of demarcation between cortex and medulla



FIG. 4—Longitudinal section of left 3rd finger showing absorption of terminal phalanx with cyst formation

examined and it was impossible to make an extensive investigation of the skeleton.

The external appearances confirmed the clinical findings. The oval cystic swellings over the left acromio-clavicular joint and over the anterior surfaces of the terminal phalanges contained thick white material of the consistency of oil paint, with intermingled chalky material. The other significant changes were limited to the genito-urinary, cardiovascular, skeletal and endocrine systems.

The *kidneys* (right 22.0 g., left 27.0 g.) showed similar appearances (fig. 3). The capsule was thickened and adherent. Both external and cut surfaces were pale. Cortex and medulla were almost indistinguishable and were stippled with numerous pale yellow areas. No calcification was evident. The vessels were not unduly prominent. The pelvis was thickened and opaque and contained no calculi. The ureters and bladder were healthy.

Heart (315.0 g.). The pericardium was healthy. The left auricle was hypertrophied, its wall being twice as thick as that of the right, which was healthy. The right ventricle (0.4 cm. thick) and tricuspid valve (13.5 cm. in circumference) were healthy. The mitral valve (8.0 cm. in circumference) was converted into a thick, rigid, stenosed ring with several irregular, projecting calcified masses. The chordæ tendineæ were neither shortened, thickened nor fused. The pulmonary valve (7.0 cm. in circumference) was slightly fenestrated and there was one small calcified nodule on the left posterior cusp. The aortic valve (6.5 cm. in circumference) showed small calcified nodules on all cusps, with slight fusion and consequent stenosis. The left ventricle showed slight muscular hypertrophy, with an average thickness of 1.8 cm. The coronary vessels were healthy. The aorta showed four calcified atheromatous plaques in the arch, otherwise it was smooth down to a point 6.0 cm. above the bifurcation, where the intima became roughened and scaly. This appearance extended into the common iliac and hypogastric arteries. The splenic artery was converted into a rigid, calcified and tortuous tube. The cervical branches of the uterine arteries were also calcified. The veins throughout appeared healthy.

Skeleton. The vertebral bodies, left clavicle, left third finger and right fourth and fifth ribs were examined. The bones were not appreciably softer than normal. The terminal phalanx of the finger showed osteoporosis with cavitation (fig. 4).

Endocrine glands. The *thyroid gland* (28.0 g.) was asymmetrical, the left lobe being larger than the right and containing a circular adenomatous nodule 2.0 cm. in diameter, with central calcification. Four *parathyroid glands* were found, each considerably enlarged,

greyish white and firm, and showing a few hæmorrhagic areas on section. Their weights were as follows:—right upper, 0.95 g.; left upper, 0.58 g.; right lower, 2.85 g.; left lower, 0.83 g.; total weight 5.21 g.

Histological examination

Kidneys. The glomeruli were reduced in number and many were sclerosed and showed partial or complete hyalinisation of the tufts; both sclerosed and surviving glomeruli were hypertrophied and relatively acellular. No fibrin nor amyloid was found with the appropriate staining. The glomerular capsules were surrounded by laminated connective tissue: there were no crescents. In the sclerosed glomeruli there were numerous small fat droplets. Many of the tubules were atrophic and enveloped in dense connective tissue; the surviving tubules were dilated, tortuous and hypertrophic. There were a few fatty and calcareous casts and some fine fat droplets surrounding the tubules. The interstitial tissue was relatively increased and showed focal accumulations of small round cells, calcareous deposits and numerous fine fat droplets. The arterioles showed slight medial thickening and intimal proliferation, with occasional small subintimal fat deposits, but no calcification. The pelvis was slightly thickened by connective tissue but showed no inflammatory changes.

The picture was one of advanced renal sclerosis but without histological evidence of chronic glomerulo-nephritis. The arteriolar changes were slight, thickening being minimal, and there was complete absence of necrotising arteriolitis, so that a diagnosis of malignant nephrosclerosis is untenable. There was no evidence of urinary infection, either gross or microscopic, and the appearances in no way suggested chronic pyelonephritis. Microscopically the appearances were similar to those seen in the surviving renal tissue of polycystic kidneys, but in this case the picture was uniform throughout the kidney and suggested a diagnosis of renal hypoplasia (fig. 5).

Parathyroid glands. All four glands showed similar appearances (fig. 6), being made up of chief cells arranged either in compact branching columns or in acinar formations. Some of the latter consisted of true acini filled with colloid-like material and in places formed cystic spaces lined by columnar chief cells. There were also large accumulations of dark oxyphil cells. There was little interstitial tissue and only a few fat vacuoles. The nuclei showed no hyperchromatism nor mitoses. Some of the arterioles showed medial calcification.

Skeleton. Sections were cut from the clavicle, rib and terminal phalanx. The periosteum of the rib was slightly thickened and composed of fibrous lamellæ without infiltrating inflammatory

METASTATIC CALCIFICATION



FIG. 5.—Photomicrograph of kidney showing hypertrophied glomeruli, one of which shows deposits of hyaline material. There is increased connective tissue and the tubules are dilated and hypertrophied. (Average diameter of glomerular tufts, $330\ \mu$)

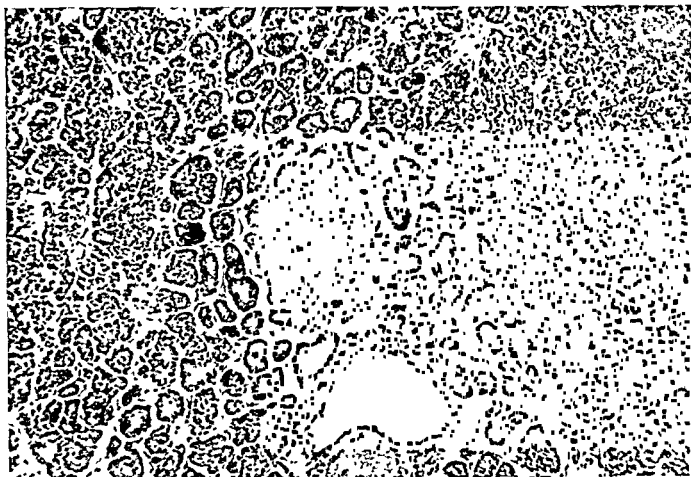


FIG. 6.—Photomicrograph of parathyroid gland showing collections of chief cells with acinar formation. In the centre of the field is a group of oxyphil cells. $\times 140$.

cells. In the other sections the periosteum was not thickened. In all sections there was considerable evidence of bone resorption, shown by numerous multinucleated giant cells lying in lacunæ at the margins of the medullary spaces, which in consequence appeared indented and scalloped. Some Haversian canals were widened and there was diminution in number of bone corpuscles, those remaining being irregularly distributed. In some areas, especially in the clavicle, there was combined resorption and apposition, the bone having an irregular arrangement, with dark-staining cement lines forming an irregular disorderly mosaic. The medullary cavity throughout was largely filled with loose fibrous connective tissue, with groups of cells, mainly lymphocytes, plasma cells and histiocytes. There were fairly numerous thin-walled capillaries but no evidence of either old or recent hæmorrhage and no aggregations of osteoclasts were seen. In a few places there was abnormal osteoid tissue without Haversian canals and with irregularly distributed bone corpuscles; this also showed evidence of resorption. Osteoblasts were not numerous in any of the sections. In one site (phalanx) there were several cysts with walls consisting, in some instances, of dense collagenous connective tissue, often indistinguishable from the surrounding fibrous marrow. Other cysts had a sharply defined even margin lined by a layer of flattened endothelial-like cells.

The main features were bone resorption with deposition of a vascular fibrous substitution tissue and very little new bone formation. Although in a few places resorption and apposition were more balanced, yet resorption was the predominant feature. The appearances were analogous to those seen in osteitis fibrosa cystica.

DISCUSSION

The outstanding features of this case are chronic renal disease, secondary hyperplasia of the parathyroid glands, decalcification of bone and calcification of other tissues, and simultaneously high concentrations of calcium and phosphate in the blood serum. Although the calcium was only slightly above the upper limit of normal, the figure is striking because the usual result of renal retention of phosphate, in the absence of parathyroid hyperplasia, is a fall in the serum calcium. If one takes the view that the concentrations of calcium and phosphate in normal plasma fall near to the saturation level for calcium phosphate, then if one of these ions rises above normal without a corresponding fall in the other the saturation level is likely to be exceeded, and the deposition of calcium phosphate in the soft tissues is just what would be expected.

Although the symptomatic history in this case covered only

four years, the condition of the kidneys suggests that the renal disease had existed for a longer period, and that the first renal symptoms were precipitated by the pregnancy. Such a view would bring the case into line with others showing similar features. Gross parathyroid hyperplasia is a rare complication of renal disease, but within the small group in which it is found, there is a high proportion of cases where the renal damage has been of long standing, and notably of congenital abnormalities of the kidney. It would seem that only in cases running a very chronic course is there time for the development of gross parathyroid hyperplasia.

Although secondary parathyroid hyperplasia of extreme degree is a rare complication of renal disease, it seems to occur more often as a result of renal disease than in other conditions. Bergstrand (1920-21, 1931) has described such cases and has stressed the finding of bony changes, which he interprets as the earliest primary lesion of osteitis fibrosa. It is noteworthy that in his earlier work in 1916-17 (quoted by Bergstrand, 1920-21) he examined the parathyroids in 200 almost consecutive post-mortem examinations and found parathyroid hyperplasia only in renal disease. In all he collected 12 cases, though in one of those published in 1920-21 the renal changes were not marked. Later work showing the association of parathyroid hyperplasia with renal disease is reviewed by Anderson (1939).

Metastatic calcification also is a rare condition, but it also has a special relation to renal disease. Barr (1932), in reviewing the subject, states that it is very difficult to find cases of metastatic calcification in which renal disease can definitely be excluded. It may occur in association with various destructive lesions of bone, but most of these are conditions in which renal damage may be a complication. Apart from renal rickets and secondary osteitis fibrosa of renal origin, in which the renal disease is primary, renal damage may occur as a complication of primary parathyroid osteitis. In multiple myeloma secondary renal damage develops in the majority of cases. This was emphasised by Geschickter and Copeland (1928) and has been confirmed in a number of cases seen in Newcastle in recent years. Destructive bone lesions other than those of parathyroid or renal origin may however be associated with metastatic calcification. We shall return to this point later.

The immediate cause of metastatic calcification is most probably an over-saturation* of the serum and tissue fluids with calcium phosphate. We believe that where renal disease is the primary abnormality, the sequence of events is usually as follows. The

* In this discussion the term "over-saturation" is used to indicate simply values above the saturation level, whatever the physical state of the calcium phosphate may be. "Supersaturation" is used in its true physico-chemical sense as a condition of unstable equilibrium.

renal failure leads to a rise in plasma phosphate. In response to the raised phosphate, the serum calcium falls. This is the common finding in the majority of cases of renal failure, and at this stage of the process bony changes of the type of renal rickets or renal dwarfism may occur in young subjects *. If the calcium deficiency lasts long enough, it stimulates hyperplasia of the parathyroids and the hyperparathyroidism mobilises calcium from the bones, either initiating osteitis fibrosa or aggravating previously existing bone disease. The serum calcium will be raised from the earlier low level to normal or even above it, but owing to the renal failure phosphate is still retained in the blood, causing over saturation of blood and tissues with calcium phosphate and so giving rise to metastatic calcification.

In studying this question we have collected from the literature cases of renal disease with parathyroid hyperplasia or metastatic calcification or both, together with three other cases of metastatic calcification associated with neither renal failure nor parathyroid overactivity (table I, p 174). We have included only cases in which blood calcium and phosphate estimations have been made. The common sites of calcification in this series were the subcutaneous tissues, heart and arteries, and to a lesser extent the lungs. Renal calcification is common, but we have not regarded this as an expression of metastatic calcification if the kidney was the only organ involved. Two cases of extreme arterial calcification in young subjects with renal rickets have been classified as instances of metastatic calcification. Calcification of the stomach, often described as part of the syndrome, is conspicuously absent.

Including our own, there are sixteen cases of metastatic calcification associated with renal disease (table I), in eleven of these there was gross parathyroid hyperplasia and in only one was parathyroid hyperplasia definitely excluded. In addition there are six cases with parathyroid hyperplasia but no metastatic calcification, and three cases of destructive lesions of bone without gross renal disease or parathyroid hyperplasia. It is at once evident that among the cases with metastatic calcification there is a large proportion in which either the serum calcium or serum phosphate is raised without a corresponding fall in the other ion, but in order to deal adequately with these cases it is necessary to consider, firstly, whether a standard can be established for the saturation levels of calcium and phosphate in serum, and secondly, the regulatory mechanisms whereby the level of blood phosphate is ordinarily lowered in response to rising calcium or blood calcium lowered in response to rising phosphate.

* The fact that bone disease may occur is absent is illustrated by the findings of renal dwarfism and renal rickets and four

Saturation of serum with calcium and phosphate

The serum calcium exists in two fractions, non-diffusible and diffusible. Only the diffusible fraction can be concerned in an equilibrium with phosphate determining the saturation level. It is well established that the non-diffusible calcium is combined with protein and that there is a simple chemical equilibrium between the diffusible and protein-bound calcium according to the law of mass action. The constants for this equilibrium have been worked out by Greenberg *et al.* (1934-35) and by McLean and Hastings (1935 *a* and *b*); their formulæ are differently expressed but give similar results. Either the nomogram of McLean and Hastings or the formula of Greenberg *et al.* can be used to calculate the diffusible from the total calcium when the serum protein level is known.

The normal range of diffusible serum calcium is from 4.5 to 6.5 mg. per 100 c.c. Since this represents a higher concentration than would be expected from the solubilities of the salts which may be present, earlier workers in this field either postulated a true supersaturation of normal serum in respect of calcium and phosphate ions, or assumed that a considerable part of the free calcium occurred in the form of a non-ionised complex. Schmidt and Greenberg (1935) in their review showed that there was no positive evidence for such a non-ionised fraction, and the subsequent work of McLean and Hastings (1935*a*) strongly supports the view that the diffusible calcium is almost all ionised. This implies that under the complex conditions in serum the solubility is greater than had been calculated from the physico-chemical data available, and that one cannot calculate the saturation level in serum by ordinary chemical methods. However, since the plasma is in chemical equilibrium with the extravascular tissue fluid and this fluid is in contact with the solid salts of bone, it seems that normal serum cannot be far below saturation point, otherwise the solid bone could not be permanently preserved. Uncertainty as to the precise solid compound involved makes it impossible to calculate the solubility product for a solution in contact with solid bone. Robison's theory of bone deposition, which has good experimental support, postulates that the phosphatase of the osteoblasts liberates inorganic phosphate in the region of the cell, thus causing local over-saturation and deposition of calcium phosphate. The theory implies that the tissue fluids are somewhat under-saturated except in the region of phosphatase action, but the mechanism fails if there is gross under-saturation, as in nutritional rickets and osteomalacia.

Further evidence that normal serum is not much below saturation level is afforded by the fact that when the concentration of either calcium or phosphate is artificially increased by the addition of

either ion, a colloidal complex containing calcium and phosphate is formed. This occurs both *in vitro* and *in vivo* (Grollman, 1927, Scholtz, 1931, Laskowski 1933, Greenberg *et al*, 1934 35, McLean and Hinrichs, 1938). The composition of this complex is not known, but it is reasonable to suppose that it represents a separation of undissociated calcium phosphate, which remains dispersed in colloidal form presumably because other colloids (proteins) prevent its aggregation as a gross precipitate. It is interesting that Gersh (1938) in making histological studies of the material of McLean and Hinrichs, observed that when the colloidal calcium phosphate complex was formed it was taken up by reticulo endothelial cells. We were unable to demonstrate deposition of calcium phosphate in the reticulo endothelial system in our case of metastatic calcification.

We have made further calculations from the data of McLean and Hinrichs and Greenberg *et al* * in an attempt to determine the corresponding saturation levels of calcium and phosphate. We have assumed that the presence of the colloidal complex is a sign of over saturation. On this view, the concentration of non colloidal calcium and phosphate in a mixture which contains the colloidal complex will be exactly at the saturation level under conditions of equilibrium. The data of Greenberg *et al* supply figures for diffusible calcium and phosphate in systems where the non diffusible complex had been formed by addition of calcium salts to serum. We have interpreted these figures for diffusible calcium and phosphate as showing saturation values directly. The data of McLean and Hinrichs give figures for ionised calcium, protein bound calcium and the calcium of the colloidal complex, and for total phosphate. These results were obtained by addition of phosphate to serum *in vivo* and *in vitro*. We have used only the data for systems containing the colloidal complex at equilibrium with the non colloidal fractions. We have calculated the non colloidal or "free" phosphate by correcting the total phosphate figures for the colloidal phosphate on the assumption that the proportions of Ca to P in the complex are those of $\text{Ca}_3(\text{PO}_4)_2$. In fact, as this is a small correction on a large total phosphate, it makes little difference whether one assumes $\text{Ca}_3(\text{PO}_4)_2$ or CaHPO_4 .

The results are striking in that when one plots free calcium against free phosphate one obtains very nearly a rectangular hyperbola corresponding to a constant $\text{Ca} \times \text{P}$ product of 30 (when Ca and P are expressed as mg per 100 c c). Fig 7 shows the points derived from the original papers and the curve corresponding to

* Only these two papers afford suitable data. Laskowski also has figures for diffusible calcium and phosphate in sera to which either calcium or phosphate was added but his ultrafiltrates were in many cases truly supersaturated and do not represent equilibrium conditions.

$\text{Ca}^{++} \times \text{P} = 30$.* It is striking that the data derived from McLean and Hinrichs, with raised phosphate, link up smoothly with those of Greenberg *et al.*, with raised calcium. The diagram also shows the zone of diffusible Ca and phosphate in normal serum, which falls only just below the saturation level.

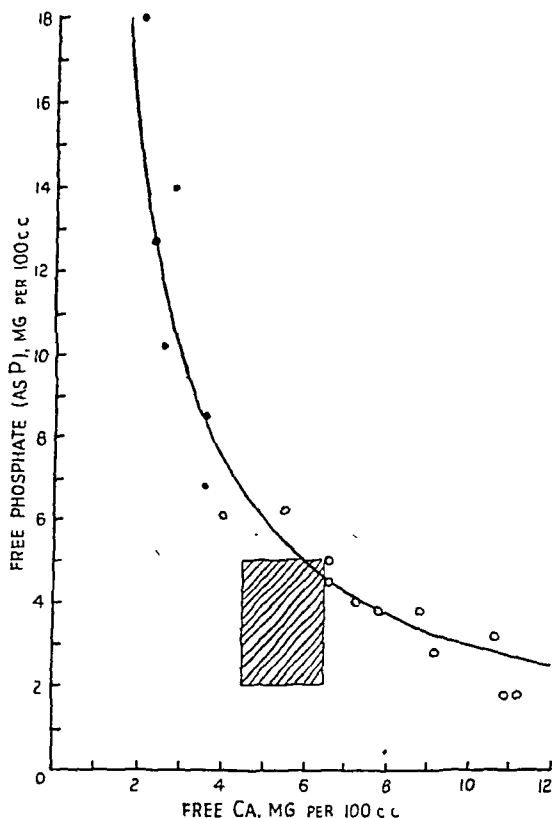


FIG. 7.—Corresponding values of non-colloidal calcium and phosphate at the saturation level.

Continuous curve = rectangular hyperbola corresponding to $\text{Ca} \times \text{P} = 30$.

Plotted points = solid circles show data calculated from McLean and Hinrichs; open circles show data of Greenberg *et al.*

Shaded area = zone of diffusible calcium and total phosphate in normal serum.

In the following discussion this saturation curve is used as a standard of reference. Where only the values for total calcium are available, we have compared them with "saturation curves" obtained by calculating, from the diffusible calcium, the corresponding total calcium levels at the extreme limits of normal serum

* If the solid were $\text{Ca}_3(\text{PO}_4)_2$ the constant should be $(\text{Ca}^{++})^2 \times (\text{PO}_4)^2$. This value is in fact not a constant. The finding of a constant value for $(\text{Ca}^{++}) \times (\text{PO}_4)$ suggests that the proportions of calcium and phosphate in the solid are those of CaHPO_4 .

protein. These standards are put forward tentatively, but they have the advantage over other formulæ in that they are based on the levels at which calcium and phosphate separate from true solution. On the other hand, they do not allow for changes in solubility at different hydrogen ion concentrations.

Reciprocal adjustment of calcium and phosphate

In hyperparathyroidism a rise in serum calcium is accompanied by a fall in serum phosphate and the reverse occurs in parathyroid deficiency. In renal insufficiency a rise in serum phosphate is associated with a fall in serum calcium. It is often stated, in general terms, that there is an "inverse ratio of calcium and phosphorus", but this description may be misleading, for if the primary abnormality is a fall in one ion, the other does not necessarily rise. The important point seems to be that so long as the regulatory mechanisms are functioning efficiently, a rise in one ion is balanced by a fall in the other, and over-saturation is avoided or minimised.

In primary hyperparathyroidism without renal disease hypercalcaemia is accompanied by low serum phosphate. The phosphate is either definitely subnormal or in the low normal range. The parathyroid hormone causes increased urinary excretion of both calcium and phosphate, a rise in serum calcium and a fall in serum phosphate and mobilisation of salts from the bones. An attempt has been made by Albright and his collaborators (Albright *et al.*, 1929; Albright and Ellsworth, 1929) to relate all these changes to a primary effect on the renal excretion of phosphate. According to this view the primary action of the hormone is to lower the renal threshold for phosphate. The increased phosphate excretion leads to a fall in the phosphate of the plasma so that it becomes unsaturated in respect of calcium phosphate and thus dissolves calcium phosphate from the bones. Mobilisation of calcium from bone raises the serum calcium and this in turn leads to increased urinary excretion of calcium. We do not accept the view that the change in serum calcium in hyperparathyroidism is secondary to the change in serum phosphate. Thomson and Pugsley (1932) have shown that a fall in serum phosphate does not in itself lead to a rise in serum calcium. We have recently observed a case of primary hyperparathyroidism in which, after parathyroidectomy, the serum calcium fell at once, whereas the plasma phosphate took several weeks to return to normal (from a pre-operative level of 0.5 mg. P per 100 c.c.). Moreover, if the decalcification of the bones were due simply to under-saturation of the plasma in respect of the bone salts, one would expect a simple decalcification such as occurs in osteomalacia, whereas in fact the anatomical lesions in hyperpara-

thyroidism indicate active participation of the bone cells. Therefore it seems that the parathyroid hormone acts directly on both bone and kidney. The occurrence of osteitis fibrosa and metastatic calcification in the secondary parathyroid hyperplasia of renal disease, when the plasma phosphate is high, can only be explained on the view that the mobilisation of calcium from the bones is due to a direct effect of the parathyroid hormone on the bones.

In fig. 8 are shown data for serum calcium and phosphate in primary hyperparathyroidism without any known renal lesion. These are derived from the cases collected by Hunter and Turnbull (1931-32), together with seven unpublished cases seen in this hospital. As serum protein levels are unknown for most of the cases, the values

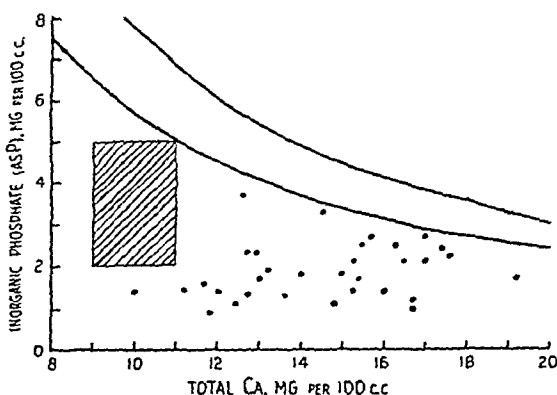


FIG. 8.—Corresponding values of total serum calcium and inorganic phosphate in primary hyperparathyroidism.

Continuous curves, upper and lower, show the saturation levels for serum protein 8.5 and 5.6 per cent. respectively.

Plotted points = cases of hyperparathyroidism.

Shaded area = zone of normal serum calcium and phosphate.

plotted are those for total calcium, and two saturation curves are given, one corresponding to serum protein 5.6 per cent., the other to serum protein 8.5 per cent. The points all fall below the saturation level. (Two cases of Duken's (1928), quoted by Hunter and Turnbull, are excluded because the original reports show that one was a case of sarcomatosis and in the other the diagnosis of primary hyperparathyroidism was not established. Both showed over-saturation.) It is evident that the regulation of phosphate in primary hyperparathyroidism is fully efficient in preventing over-saturation of the serum in the absence of gross renal insufficiency.

The mechanism whereby the serum calcium is lowered in response to the raised phosphate in severe renal insufficiency (in the absence of gross parathyroid hyperplasia) is not well understood, but it seems that an increase in faecal excretion of calcium phosphate is an important factor. Although phosphate is increased in the blood,

there is little or no retention of phosphate in the body as a whole; phosphate balances are sometimes slightly positive, sometimes actually negative, the excess of excretion over intake being accounted for by faecal excretion. The evidence is summarised by

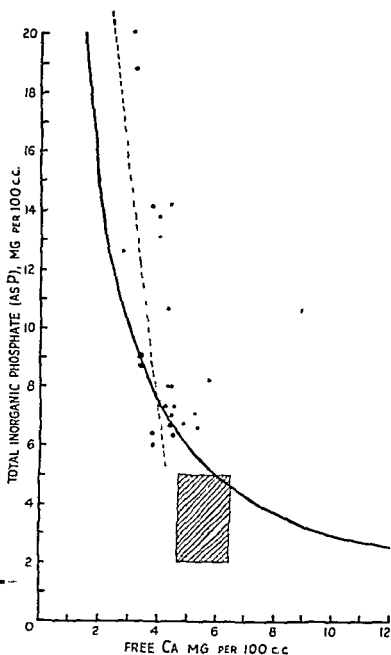


FIG 9.—Corresponding values for diffusible calcium and total inorganic phosphate in renal failure.

Continuous curve = saturation level, $\text{Ca} \times \text{P} = 30$.

Dotted line = formula of Peters and Eiserson, based on cases of renal disease

Plotted points = cases of Herbert and Salvesen and Linder

Shaded area = zone of diffusible calcium and total phosphate in normal serum.

Mitchell (1930) and Mitchell and Guest (1938). The increase in faecal excretion of phosphate favours precipitation of calcium phosphate in the gut, and this is probably a factor in lowering the serum calcium. Whether this is regarded as a truly physiological regulation or not, it seems to be a relatively inefficient means of preventing over-saturation of the serum, especially when the serum phosphate is very high. In fig. 9 are shown data for diffusible

TABLE I
Cases of secondary parathyroid hyperplasia or metastatic calcification or both collected from the literature

Case no.	Author	Diagnosis	Parathyroids	Metastatic calcification	Ca, mg. per cent. (serum)	P, mg. per cent. (serum or plasma)	Urea,* mg. per cent. (blood)	NPN, mg. per cent. (blood)	Protein per cent. (serum)
1	Gilligan <i>et al.</i> (1938)	Chronic nephritis	Slight hyperplasia	None mentioned	6.7	8.6	...	126	6.0
2	"	"	"	"	8.4	7.0	...	96	5.8
3	"	"	"	"	4.0-6.4	9.0-8.1	...	195-131	5.1-5.8
4	"	Renal rickets	"	"	8.5	5.8, 7.0	...	97, 126	6.2, 6.7
5	Price and Davio (1936-37)	Renal rickets : osteoporosis	Hyperplasia	Absent	13.6-12.5	6.5-5	318-397
6	Dukon (1928)	Renal rickets : bilateral hydronephrosis	Slight hyperplasia	"	10.1-16.0	9.0-6.0	...	107	7.27
7	Present case	Renal hypoplasia : decalcification of bone	Hyperplasia	Extensive	11.7	7.8	300
8	Pollack and Siegal (1935-36)	Chronic nephritis : no gross bone disease	"	"	12.13	4.6-3	206
9	Smyth and Goldman (1934)	Renal rickets	"	Present	10.5-11.9	10.9-16.0	...	80-333	6.0
10	Shelling and Remsen (1935)	"	"	Arterial only	9.6	8.0	...	153-364	...
11	Castelman and Mallory (1935)	Chronic pyelonephritis : decalcification of bones	"	Present	8.0	9.0	...	150	...
12	Castelman and Mallory (1937)	Chronic nephritis : secondary osteitis fibrosa	"	"	10.1	7.92	...	120	4.9

calcium and total phosphate in renal failure. The points plotted are taken from the papers of Salvesen and Linder (1923-24) and Herbert (1933). Diffusible calcium was determined in Herbert's work and has been calculated from the total calcium and protein figures of Salvesen and Linder. In addition the formula of Peters and Eiserson (1929) has been utilised, and the dotted line in the graph shows their results calculated in terms of the relation between diffusible calcium and phosphate. The data were derived from cases of renal disease with raised phosphate. It will be seen that with serum phosphate in the region of 6-9 mg. per 100 c.c. the levels of calcium and phosphate in these cases usually fall slightly below or slightly above the saturation curve, but with greater rises in phosphate the apparent saturation level is consistently exceeded. It must be remembered, however, that the genuine saturation level may be raised in such cases by acidosis. This may explain the absence of clinical evidence of gross calcification in this type of case. Moreover, in many of these cases the over-saturation may have been a terminal condition of short duration, though this is not always so; some of Herbert's cases showed apparent over-saturation for long periods. It is possible that tissue analyses in cases of this type might show evidence of increased calcium in advance of gross calcium deposition. Although gross metastatic calcification is absent in cases in which the apparent saturation level is exceeded, it is of interest that this apparent over-saturation is common in renal failure, whereas it is not found in primary hyperparathyroidism without renal disease.

In most cases of renal rickets the serum calcium falls in response to a rise in serum phosphate, but not invariably. Parsons (1927) records some instances in which both serum calcium and serum phosphate were high, and the serum was presumably over-saturated, without any record of metastatic calcification.

Analysis of published cases

We return now to the collected cases in table I. The first six are instances of renal disease with secondary parathyroid hyperplasia but without metastatic calcification. In the four cases of Gilligan *et al.* (1938) the parathyroid hyperplasia was of the slight degree commonly met with in chronic nephritis (Pappenheimer and Wilens, 1935), and the rise in serum phosphate was associated with a lowering of the serum calcium sufficient to bring the blood levels below saturation point. In our view these cases represent a stage at which the parathyroids were responding to the stimulus of hypocalcæmia but had not yet reached sufficient activity to raise the serum calcium to the saturation level. In the case of Price and Davie (1936-37) there was gross parathyroid hyperplasia and the

blood calcium was above normal, the blood was probably slightly over saturated but there was no metastatic calcification. Duken's case is similar, showing apparent over saturation without metastatic calcification, but here there was a severe acidosis.

The remaining nineteen cases all show metastatic calcification. In seventeen, figures for both serum calcium and serum phosphorus are available. As several have no figures for serum protein, we

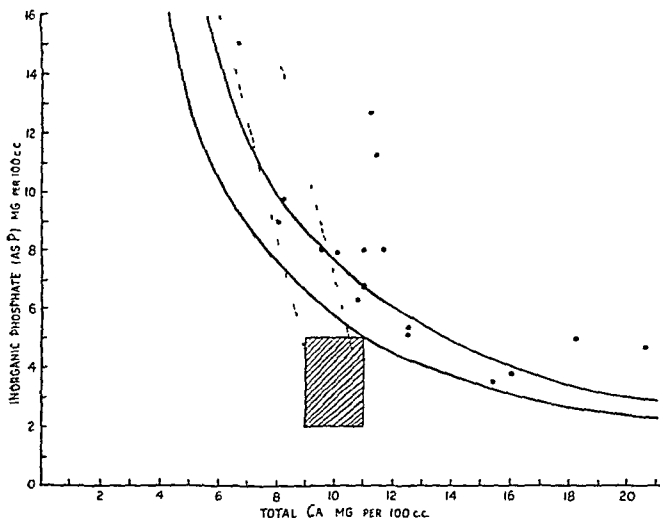


FIG. 10—Corresponding values of total calcium and inorganic phosphate in metastatic calcification.

Plotted points = cases of metastatic calcification.

Continuous curves, upper and lower, show saturation levels at serum protein 8.5 and 5.6 per cent respectively.

Dotted lines correspond to the formula of Peters and Emerson at serum protein 8.5 and 5.6 per cent. They indicate approximately the region of corresponding Ca and P figures in ordinary cases of renal failure.

Shaded area = zone of normal calcium and phosphate.

have plotted in fig. 10 the values for total calcium and phosphate, together with the "saturation curves" corresponding to the extreme limits of normal serum protein (5.6 and 8.5 per cent). It will be seen that no points fall below the lower curve and all but six fall on or above the upper. Among the renal cases it is known that some had subnormal serum protein and it may safely be assumed that none would have raised protein (except case 22, where the

primary condition was multiple myeloma). It is therefore safe to assume that the points on or above the upper curve indicate over-saturation according to our standard. Further details are of interest in regard to the six points falling between the two curves. In three of these, protein figures are available (cases 17, 20 and 24). The point plotted for case 17 is a mean of several determinations, some of which showed over-saturation and others levels below saturation. In cases 20 and 24 the level is slightly above saturation when the protein figure is taken into account (see table II). In case 8 the point plotted is the mean of two pairs of analyses, one of which would have fallen above the upper curve of fig. 10. So in these four cases the saturation level was exceeded, at least temporarily. This leaves only two cases (11 and 22) in which it is uncertain whether our empirical saturation level was exceeded or not. Both would be supersaturated if the serum protein was in the low normal range or subnormal.

In table II are shown more details of the cases in which serum protein figures are available. In all, the product $\text{free Ca} \times \text{P}$ exceeded 30 at some time.

TABLE II

Cases of metastatic calcification in which full blood analyses are available. (Numbering of cases as in table I)

Case no.	Total serum protein (per cent.)	Phosphate, mg. P per 100 c.c.	Total Ca, mg. per 100 c.c.	Free Ca, mg. per 100 c.c. (calculated)	Product, free Ca \times P
9	6.0	12.0	11.7	6.1	55
12	4.9	7.9	10.1	5.7	45
17	6.56 to 6.98	4.2 to 7.8	9.4 to 12.4	4.3 to 6.0	22 to 40
18 (a) *	7.5 to 7.7	4.0 to 6.7	9.8 to 11.3	4.2 to 4.9	17 to 32
(b) †	6.1 to 7.0	8.0 to 9.2	10.1 to 12.1	4.7 to 6.4	38 to 54
20	4.66	5.3	12.5	7.7	41
22	6.22	4.7	20.6	12.2	57
23	4.67	3.5	15.6	10.1	35
24	5.8	5.0	18.2	11.2	56
	5.5	4.1	18.7	11.8	48

* Before onset of subcutaneous calcification.

† Metastatic calcification well established.

We shall now consider the conditions associated with metastatic calcification in more detail. In cases 7-16 the primary abnormality is renal disease and there is gross secondary parathyroid hyperplasia with over-saturation of the serum and metastatic calcification. In cases 14 and 15 phosphate figures are not available but, as the calcium is either at the normal maximum or raised, it is probable that these resemble the cases in which full data are available. In cases 11 and 13 the serum calcium is reduced, but not sufficiently to avoid over-saturation. In cases 17 and 18 the parathyroids were

not examined, but otherwise these resemble the group with parathyroid hyperplasia and metastatic calcification and show normal or raised serum Ca with raised phosphate, causing over-saturation. In cases 19 and 20 there was a similar syndrome, except that the parathyroids were searched for and no hyperplasia found; it is possible, however, that parathyroid hyperplasia may have been missed. In Lightwood's (1932) case (no. 19) only one parathyroid gland was examined. In Brown and Ginsberg's case no. 20 (1940) no parathyroid tissue was found in the normal site, so it is not unreasonable to suppose there was some ectopic hyperplastic parathyroid tissue which was not found.

Among the group with secondary parathyroid hyperplasia and metastatic calcification the case of Bass and Pakter (1938) is of special interest. These authors give a series of blood analyses before and after the appearance of subcutaneous calcium deposits. Only those made during the period of metastatic calcification are shown in table I, but the complete series shows blood figures below or at the saturation level in the early stage of the case, and definitely above the saturation level during the stage of gross metastatic calcification (see table II, case 18).

The case of Platt and Owen (1934) falls in a different category from those so far described. Here there is a gross increase in phosphate and a low serum calcium with normal parathyroids. The point in fig. 10 corresponding to this case falls in the region of the formula of Peters and Eiserson—that is to say, the serum is over-saturated by our standard, but not more so than in many ordinary cases of renal failure. This is an instance of over-saturation due simply to phosphate retention inadequately compensated by the fall in serum calcium, and the metastatic calcification seems to be due to this alone, without any parathyroid overactivity.

The case of Barr and Bulger (1930) gives a point falling in the marginal zone between the two curves of fig. 10. The serum would be over-saturated if the serum protein was low or in the low normal range, but since the primary disease was multiple myeloma, it cannot be assumed that the serum protein was normal. This case cannot be satisfactorily interpreted without the serum protein figure.

Cases 23 and 25 are instances of metastatic calcification associated with destructive lesions of bone. In both, the kidneys were normal and both show over-saturation of the blood due to the association of high serum calcium with normal phosphate (see table II). Here it seems that rapid bone destruction liberated calcium and phosphate faster than they could be excreted and, as the level of parathyroid activity was normal, there would presumably be no lowering of the renal threshold for phosphate to assist its clearance from the blood. In case 24 the primary lesion is not known. The renal damage was not sufficient to cause phosphate retention and over-

saturation of the serum is due to the coincidence of high serum calcium with normal serum phosphate.

We have considered also a case reported by Durham (1928). There was generalised calcification with chronic nephritis and scleroderma; the parathyroid glands were normal and the serum calcium was 7.6 mg. per 100 c.c., phosphate 4.77 mg. P per 100 c.c. and non-protein nitrogen 60 mg. per 100 c.c. The author reported it as a case of calcinosis universalis and in view of the association with scleroderma this is probably the correct diagnosis. The blood calcium and phosphate fall well below the saturation level, and if the case were regarded as metastatic calcification it would be the one clear exception to the rule that in metastatic calcification the serum is over-saturated. This is probably an instance of the chance association of calcinosis universalis with renal disease and has been excluded from the series with metastatic calcification.

From a consideration of the whole series we may say that although apparent over-saturation of the blood may occur without metastatic calcification, metastatic calcification never occurs without over-saturation. Where renal disease is the primary abnormality, the over-saturation and metastatic calcification may occur simply from an increase in phosphate insufficiently compensated for by falling calcium. When there is also gross parathyroid hyperplasia tending to raise serum calcium, over-saturation is inevitable and metastatic calcification is likely to occur. In destructive bone lesions without renal disease, over-saturation and metastatic calcification may be due to a rise in serum calcium without a corresponding fall in phosphate.

SUMMARY

1. A case is described showing the syndrome of chronic renal disease, secondary parathyroid hyperplasia, decalcification of bones and metastatic calcification. Both serum calcium and serum phosphate were above normal. Cases in the literature are reviewed.

2. A tentative standard for the saturation levels of serum calcium and serum phosphate is put forward and used as a criterion in interpreting the blood analyses in various conditions.

3. In primary hyperparathyroidism without renal disease the fall in serum phosphate is adequate to compensate for the rise in calcium and over-saturation is avoided.

4. In renal failure without parathyroid hyperplasia the rise in plasma phosphate is associated with a fall in serum calcium, but this is not always sufficient to prevent apparent over-saturation.

5. In long-standing chronic renal disease secondary parathyroid hyperplasia may develop, presumably in response to the low serum calcium. This parathyroid activity raises serum calcium

towards or even above normal, while the phosphate remains high. Over saturation results and metastatic calcification occurs in the majority of such cases.

6 In destructive lesions of bone, over saturation of the blood and metastatic calcification may be due to a rise in serum calcium without any fall in serum phosphate.

7 Metastatic calcification seems to occur only in cases showing over saturation of the serum in respect of both calcium and phosphate.

We wish to express our thanks to Dr F J Nattrass for permission to publish the clinical record of the case here reported and to Dr Whately Davidson for the radiological reports.

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THE EFFECTS OF SULPHANILAMIDE, SULPHATHIAZOLE AND SULPHAPYRIDINE ON THE DEVELOPMENT OF GRANULATION TISSUE AND THEIR TOXIC ACTION ON STRIPED MUSCLE

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(PLATES XII-XV)

THE widespread use of chemotherapeutic drugs introduced in powdered form into wounds as a prophylactic against wound infection has rendered desirable knowledge as to their effects on the tissues themselves and on the normal healing processes. Russell and Falconer (1940) have already shown that small quantities of sulphanilamide and sulphapyridine inflict no appreciable damage when applied in powdered form to the brain, while Key and Burford (1940) report that the inhibitory effect of powdered sulphanilamide applied locally to healing fractures in dogs is slight or negligible. On the other hand Bricker and Graham (1939), utilising the technique of Harvey (1929), by which the fibroblastic response in a wound is estimated from the tension necessary to rupture it, have shown that the oral administration of sulphanilamide to dogs with experimental wounds results in a marked inhibition of fibroblastic response, especially between the third and fifth days of healing. Similarly, Wolff and Julius (1939), in a study of the effects of sulphanilamide on fibroblasts in tissue culture, have demonstrated that in concentrations of the drug exceeding 3 parts per 1000, definite injurious effects are obtained. Although it is impossible for the concentration in the blood to exceed this level following a local application (Hawling, 1940), nevertheless the concentration in the serum exuding from the wound may reach 6.6 parts per 1000 (Jensen *et al*, 1939). The possibility therefore of tissue injury and fibroblastic inhibition *in vivo* merits further investigation.

Technique

The estimation of the fibroblastic response in a wound by enumeration of the fibroblasts offers considerable technical difficulty, mainly in determining the anatomical limits within which the count should be made. This difficulty was largely overcome by utilising, instead of a simple wound, the development of granulation tissue which takes place around any piece of blood clot inserted between fascial planes, the latter making readily definable landmarks between which the fibroblasts could be counted.

The material for this study was the granulation tissue developing around discs of rabbit blood clot introduced into the subcutaneous tissue of that animal. In the rabbit's skin (fig. 1) there is a natural plane of cleavage

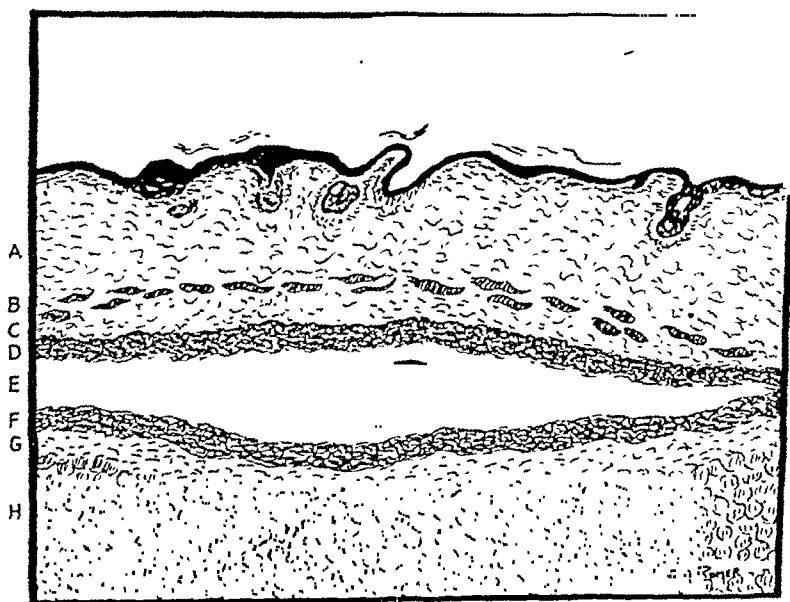


FIG. 1.—Section through rabbit's skin and subcutaneous tissues of rabbit showing blood clot *in situ*.

- | | |
|---|-------------------------------------|
| A Subcutaneous connective tissue. | E Natural plane of cleavage. |
| B Platysma. | F Granulation tissue deep to clot. |
| C Fascial plane deep to platysma. | G Fascia covering deep musculature. |
| D Granulation tissue superficial to clot. | H Muscle. |

between the dense fibrous layer deep to the platysma and a similar sheet covering the deep musculature. An incision about 2 cm. in length was made in the skin of the haunch and a pair of blunt-pointed scissors introduced to form a pocket in the natural plane of cleavage between the skin and the deeper structures. On the right side 0.25 g. of the substance under investigation was introduced, followed by a disc of rabbit blood clot 1 cm. in diameter and about 3 mm. thick. The wound was then closed by interrupted thread sutures. A similar operation was performed on the left side but omitting the introduction of the drug. Controls without drug on either side were also studied. The clot used was of blood withdrawn about 3.5 hours previously and from which the bulk of the serum had already become expressed. Animals were killed after the operation at intervals of 1, 2, 3, 5, 7 and 14 days. The clot and surrounding tissues were excised and fixed in

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FIG. 2.—Section of a 7 days-old wound treated with sulphapyridine, showing the clefts in which the drug was still present. Note the invasion by fibroblasts of the spaces between the solid particles of the drug. H and E. $\times 330$.



FIG. 3.—Section of a 24-hours old wound treated with sulphanilamide, showing marked necrosis of several layers of muscle fibres. H. and E. $\times 85$.

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FIG. 4.—Section of a 5 days old wound treated with sulphanilamide, showing hyaline degeneration of muscle fibres and invasion of the muscle by granulation tissue. H and E $\times 85$



FIG. 5.—Section of a 7 days old wound treated with sulphanilamide, showing extensive invasion of the injured muscle by granulation tissue. H and E $\times 85$

ACTION OF SULPHONAMIDES ON TISSUES

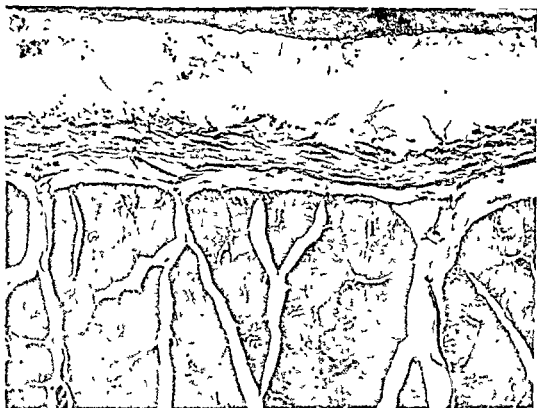


FIG 6.—Section of an untreated 24 hours old wound, showing only slight edema of the muscle tissue H. and E $\times 85$



FIG 7.—Section of an untreated 7 days old wound, showing healthy muscle and a wide zone of granulation tissue between it and the blood clot H. and E $\times 85$

ACTION OF SULPHONAMIDES ON TISSUES

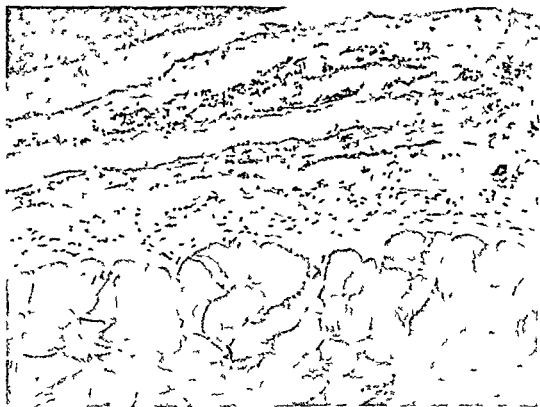


FIG 8—Section of a 24 hours old wound treated with sulphathiazole showing a zone of coagulated exudate and polymorphonuclear leucocytes and slight oedema of the neighbouring muscle H and E $\times 85$

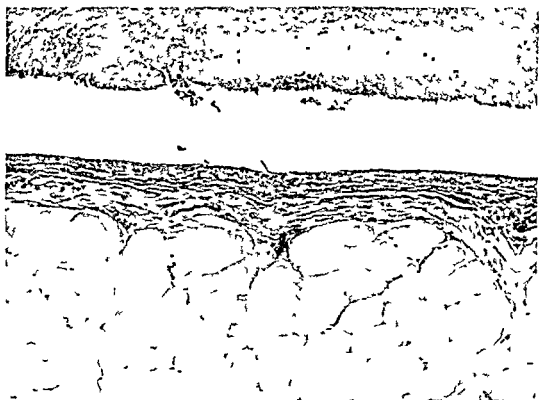


FIG 9—Section of a 7 days old wound treated with sulphathiazole showing a narrow band of granulation tissue between the clot and the healthy muscle H and E $\times 85$

10 per cent formol saline. Animals in which the wounds became septic were discarded. The material was embedded in paraffin, sections were cut 7μ thick and stained with Ehrlich's acid haematoxylin and eosin, Masson's haematoxylin, fuchsin ponceau and light green and Laidlaw's reticulum stain. It was found that the most satisfactory method of comparing the granulation tissue development was by counting the fibroblasts present in strips of standard width extending from the superficial to the deep fibrous sheath inclusive and passing through the disc of clot. The count was performed at a magnification of 440 diameters, the width of the strip being kept constant by the introduction into the eyepiece of an obturator with a fixed linear gap. Care was taken to cut the sections at right angles to the surface of the clot and to choose sections near the centre of the clot on which to make the count. In each case the figures given are the mean of at least five such counts.

Results

Qualitative effects of the drugs on the development of granulation tissue are slight. In each case during the first 3 days oedema and polymorphonuclear cell emigration were more marked on the treated side but neither was excessive. In the animals treated with sulphathiazole, crystals of the drug were visible both macroscopically and microscopically as late as 5 days after the operation. Sulphapyridine was still present in solid form after 7 days, whilst sulphanilamide had completely dissolved at 24 hours. In no case was there any giant cell reaction to the presence of the crystals, even when these had been present for 7 days. The macrophage response in these wounds, roughly estimated from the degree of invasion of the introduced blood clot, did not appear to be impaired, although phagocytic activity as shown by intracellular haemosiderin was distinctly less in the treated than in the untreated wounds. In this respect no significant difference was noted between the three drugs investigated. The development of new blood vessels varied within wide limits but the variations showed no correlation with the treatment and no inhibitory effect could therefore be assigned to any of the compounds. No qualitative differences were detected in the appearance of collagen or pre collagen fibres as a result of any of the drugs investigated.

The quantitative effect on granulation tissue formation as determined by fibroblast counts at 5, 7 and 14 days is shown in table I. The difference between the proliferation of these cells in the control animals and in those treated with sulphanilamide are not statistically significant. The differences at 7 days between the control animals and those treated with either sulphathiazole or sulphapyridine are on the border line of what is usually accepted as significant. This remarkably slight inhibitory action is well illustrated in fig 2, in which proliferating fibroblasts can be seen invading the spaces between the crystalline particles of the drug. The absence of any apparent fibroblast inhibition in the presence

of sulphanilamide is probably due to the stimulating effect of injured muscle tissue (*vide infra*).

TABLE I

Relative mean cell counts of granulation tissue in wounds subjected to the local influence of sulphanilamide and its derivatives

Days after operation	Controls			Sulphanilamide			Sulphathiazole			Sulphapyridine		
	No. of animals	Mean cell count	σ	No. of animals	Mean cell count	σ	No. of animals	Mean cell count	σ	No. of animals	Mean cell count	σ
5	4	73	12.1	2	42.5	0.5	2	47	11
7	6	101	10.0	4	92.5	34	4	56	8.2	4	71	4.4
14	6	131	54.0	2	108	14	3	104	10.0

Table II summarises the findings as regards injury to the underlying striated muscle in these experimental wounds. This

TABLE II

Injury to voluntary muscle by sulphanilamide and its derivatives

Controls				Sulphanilamide				Sulphathiazole				Sulphapyridine			
Lab. no.	Days after operation	Degree of muscle injury		Lab. no.	Days after operation	Degree of muscle injury		Lab. no.	Days after operation	Degree of muscle injury		Lab. no.	Days after operation	Degree of muscle injury	
		R.	L.			R.	L.			R.	L.			R.	L.
31	1	—	—	53	1	++	—	25	1	—	—	62	1	++	±
32	1	—	—	54	1	++	—	26	1	—	—	63	3	—	+
38	2	±	—	50	5	+	—	27	2	—	—	64	7	++	++
40	3	—	—	51	5	++	—	28	2	—	—	65	7	—	—
41	3	—	±	47	7	++	+	29	3	—	—	66	7	—	—
33	5	—	—	48	7	++	+	30	3	—	—	67	7	±	—
39	5	—	—	52	7	++	—	21	5	—	—
34	7	—	—	61	7	++	—	22	5	—	—
35	7	—	—	49	14	±	—	23	7	—	—
56	7	—	±	59	14	—	—	24	7	—	—
36	14	—	—	57	7	—	—
37	14	—	—	60	7	—	—
55	14	—	—	44	14	—	—
...	45	14	—	—
...	58	14	—	—

± = a few injured fibres.

+ = injury of superficial 2-3 layers of muscle fibres.

++ = injury more extensive and involving a depth of 5 or 6 muscle fibres.

R. = treated side, L. = control.

injury is never extensive: at most it affects the superficial 5 or 6 layers of muscle fibres. It will be seen from the table that all the cases treated with sulphanilamide and examined within 7 days showed definite muscular injury on the treated side (R.). At 24 hours

(fig. 3) this showed itself by swelling, loss of striation, fragmentation and slight cellular reaction. The later examples (figs. 4 and 5) showed invasion of the injured tissue by macrophages and granulation tissue (cf. figs. 6 and 7, from control wounds). Of the six cases treated with sulphapyridine distinct muscular injury was present in three. Of the 15 cases treated with sulphathiazole not one showed evidence of muscular injury (figs. 8 and 9) apart from some œdema at 24 hours, and of the 26 wounds in 13 control animals a slight degree of injury was present in 2 cases.

Conclusions

Sulphanilamide, probably owing to its greater solubility, is slightly but definitely more injurious to voluntary muscle than either sulphathiazole or sulphapyridine. The latter, despite its very slight solubility, is more toxic to muscle than the former. The difference therefore from drug to drug is not entirely a matter of solubility. This result conforms with the known lack of complete correlation between solubility and bacteriostatic action. It must be emphasised that the actual amount of muscle injured is in any case slight and in no way contra-indicates the local application of these drugs to flesh wounds. At most it might be suggested that in wounds of the face, hands and forearms, where the minimum of injury and scarring is desirable, sulphathiazole be given preference over the other compounds.

As regards the inhibitory action of these drugs on the proliferation of fibroblasts, the effects are so slight that investigation requiring a large number of animals would be necessary to establish really significant differences. From the small number of animals used here it is nevertheless justifiable to conclude that any inhibitory action is too slight to affect materially the healing of any wound submitted to their local application.

Summary

1. The effects of the local application of powdered sulphanilamide, sulphathiazole and sulphapyridine on the development of granulation tissue have been studied.

2. A slight, barely significant inhibition of fibroblast proliferation is produced by sulphathiazole and sulphapyridine but not by sulphanilamide.

3. Sulphanilamide and to a lesser extent sulphapyridine have a slight but definite toxic action on striped muscle.

4. Neither the fibroblastic inhibition nor the toxic action on muscle is sufficient to contra-indicate the local application of these drugs.

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THE GROWTH OF COLIFORM BACILLI IN
DISTILLED WATER

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A PREVIOUS paper (Bigger, 1937) recorded the growth of various types of coliform bacilli in a variety of natural and sand-filtered waters. Only rarely were the bacilli found to grow in raw water but growth frequently occurred in water which had been autoclaved, boiled or filtered through Pasteur-Chamberland (F) candles. These observations have been confirmed by a number of other workers. The late Col. C. H. H. Harold (1937), however, did not confirm the reported effects of filtration, his final conclusion being that "candle filtration neither appreciably reduces the growth-promoting qualities of boiled river-derived water, nor does it induce such qualities in raw water". Harold attributed Bigger's results to the contact of water with rubber tubing during filtration. He stated that filtration of distilled water, when rubber tubing was used, occasionally rendered it growth-supporting and also that distilled water in which certain specimens of rubber tubing had been boiled supported the growth of coliform bacilli.

It was primarily to clear up the dispute about filtration that the investigation now recorded was commenced, but during the progress of the work discoveries were made which appeared to be of much greater importance than the disagreement which originated it, and it is with these that the paper is mainly concerned.

METHODS

Unless otherwise stated, 240 c.c. of water, with or without the additions mentioned, were used in a 500 c.c. flask. Where the water had been filtered or treated otherwise than by heat, this amount was measured into the flask. Where the water was to be boiled or autoclaved, 250 c.c. were introduced into the flask and treated in it. The result was a reduction in volume to about 240 c.c. After cooling, if necessary, the water was inoculated by adding to it 10 c.c. of a 1 : 100,000 dilution (in distilled water) of a twenty-hour broth culture of the organism tested. The contents were mixed by rotating the flask, which was then incubated at 37° C. unless otherwise stated. Counts of the living organisms present were made from time to time by removing and plating in agar. For the majority of the experiments the same coliform bacillus (*bact. E*) was used as in the earlier work.

EXPERIMENTAL OBSERVATIONS

Experiments on filtration

Experiment 1. Five Chamberland filters were mounted in the usual way with rubber connections. Filtrates of distilled water from each filter were inoculated with bact. E (250 per c.c.) and incubated. In three cases counts of 0 were obtained on the first and subsequent days. In one case the count fell slowly to 0, which was reached on the sixth day. The fifth filtrate gave counts increasing to 3,000,000 per c.c. on the fourth day. It was noted that the rubber tubing used to connect the fifth filter to the receiving flask was new and that the filtrate contained a fine white powder in suspension.

The one positive result confirmed Harold's finding that distilled water filtered through a porcelain filter mounted with rubber connections may be capable of supporting the growth of coliform bacilli.

It now became necessary to determine if filtration of natural waters rendered them growth-supporting merely because they had been in contact with rubber. With this object in view, filters were mounted by the following method.

The end of the glass tube connecting the candle to the receiving flask was drawn into a capillary. This was inserted through the nipple into the interior of the candle. The junction between the nipple and the glass tube was luted with sealing-wax to give an air-tight junction. No water came in contact with the sealing-wax but, as a control, distilled water in which sealing-wax had been boiled was inoculated with bact. E and incubated; no growth occurred. This method of mounting filters secured that water which was filtered had been in contact only with the filter itself and with glass.

Experiment 2. Two portions of a sample of tap water were filtered through the same candle. The first filtration was carried out without, the second with rubber connections. Two other

TABLE I
Growth of bact. E in tap water

Day	Tap water					
	Raw	Boiled	Filtrates from filter 1		Filtrates from filter 2	
			Without rubber	With rubber	With rubber	Without rubber
0	280	280	280	280	280	280
3	0	0	400	0	340,000	1,000,000
5	0	0	4000	130	50,000	400,000
7	0	0	270,000	100,000	30,000	390,000
10	0	0	36,000	27,000	29,000	180,000

In this and subsequent tables, the figures give the number of bacteria per c.c. capable of growing in nutrient agar.

portions were filtered through a second candle, rubber connections being used for the first filtration but not for the second. The usual raw and boiled controls of the same sample were set up and all were inoculated with bact. E, and incubated. The results are shown in table I.

The water, both raw and boiled, failed to support the growth of bact. E, but the organism grew in the water after filtration. Filtration without rubber connections appeared to be more effective in making the water growth-supporting than did filtration with rubber connections.

Experiment 3. Both distilled water and tap water were used for this experiment. The distilled water was tested after boiling, the tap water both raw and after boiling. A portion of the distilled water was filtered through a Chamberland filter (no. 11) mounted without rubber connections. Some of the tap water was filtered through the same filter, which received no treatment between filtrations. A similar filter (no. 12), without rubber connections, was used for the filtration of tap water and subsequently, without treatment, of distilled water. The results are recorded in table II.

TABLE II
Growth of bact. E in distilled and in tap water

Day	Distilled and tap water						
	Distilled water, boiled	Tap water		Filtrates from filter 11		Filtrates from filter 12	
		Raw	Boiled	Distilled water	Tap water	Tap water	Distilled water
0	300	300	300	300	300	300	300
2	21	62	7600	0	150,000	180,000	0
5	0	0	600	0	76,000	85,000	0
7	0	0	340	0	120,000	110,000	0

The raw tap water was incapable of supporting growth. After boiling, it supported growth of bact. E, but only to a small extent. Its growth-supporting properties were greatly improved by filtration so conducted that the water was never in contact with rubber. Filtration of distilled water through the same filters did not render it growth-supporting.

These experiments establish beyond dispute that a natural or sand-filtered water which, in its raw state, is not growth-supporting, may acquire the property of supporting the growth of a coliform bacillus as a result of being filtered through a Chamberland filter even when all contact with rubber has been eliminated.

It is believed that Bigger's conclusion that filtration of natural and sand-filtered waters renders them growth-supporting by removing from them growth-inhibiting substances is valid.

Experiments with rubber

Harold's finding that distilled water became capable of supporting the growth of coliform bacilli when rubber was boiled in it was next investigated.

Experiment 4. Nine varieties of rubber tubing were tested by boiling two inches of each in 250 c.c. of distilled water for about five minutes, at the end of which time the tubing was removed. When the water was cold it was inoculated with bact. E and incubated at 37° C. In six cases no growth of the bacterium occurred, but in three there were increased counts. The most marked of these was an increase from 380 per c.c. to 1,200,000 per c.c. in three days.

This experiment showed the correctness of Harold's observation that, in some cases, by contact with rubber tubing while boiling, distilled water acquired growth-supporting qualities. It was observed in one flask in which growth occurred that the water was turbid owing to the presence of a fine white powder. A similar appearance had been observed in the filtrate which gave growth in experiment 1. Enquiry showed that this was probably talc or french chalk, commonly used as a surface dressing for rubber tubing.

Experiment 5. This experiment, which confirmed a number of others, shows that the effect of rubber on distilled water is due, not to the rubber or to a combination of rubber and talc, but solely to the talc (table III).

TABLE III

The effect of talc on the growth of bact. E in distilled water

Day	Distilled water				
	Boiled	Boiled with new rubber tubing	Boiled with cleaned new rubber tubing	Boiled with cleaned new rubber tubing plus talc	Boiled with talc
0	380	380	380	380	380
2	0	540,000	0	800,000	1,000,000
4	0	100,000	0	750,000	440,000
7	0	80,000	0	250,000	67,000

On scores of occasions distilled water, autoclaved or boiled in a flask with talc, has been inoculated with bact. E. When the flask has been plugged with cotton wool and incubated in air at 37° C.,

growth has never failed to occur, a few hundred per c.c. increasing within 2-3 days to several hundred thousand or, on occasion, to over one million.

The growth-promoting substance, which Harold stated "might be derived from sterilised rubber connections", has now been shown to be talc.

Experiments with talc

Talc is hydrated magnesium silicate and is therefore incapable of supplying the bacteria with either the nitrogen or the carbon required for their growth. Since it was thought possible that the talc used might be mixed with some impurity containing either nitrogen or carbon or both, it was submitted to various treatments designed to purify it. These included repeated washing in distilled water, boiling in distilled water, roasting in a furnace and prolonged treatment with concentrated sulphuric acid. Despite these, alone or in combination, the talc, when added to distilled water, rendered it capable of supporting the growth of coliform bacilli. It therefore appears to be legitimate to conclude that, although the addition of talc to distilled water converts it into a culture medium, the talc does not itself supply the necessary nutritive materials.

In searching for an alternative source of nutriment we are forced to consider the distilled water used. For all the work recorded, doubly distilled water was employed. Tap water was first distilled in a metal still. The distillate, to which a small amount of potassium permanganate was added, was re-distilled in an all-glass Pyrex still. All flasks used were thoroughly cleaned and rinsed with this distilled water before use. Very many samples of distilled water were tested without the addition of talc and on no occasion did growth occur.

Next we must consider the inoculum. In all the experiments except where stated, 10 c.c. of a 1:100,000 dilution in distilled water of a 20-hour broth culture of bact. E were added to approximately 240 c.c. of water. The dilution of broth in the water tested was therefore 1:2,500,000. It is highly improbable that broth in this dilution would act as a nutrient and, in fact, it was ascertained (Bigger, 1937) that the highest dilution of peptone in distilled water in which bact. E grew was 1:5000.

Experiment 6. In order, however, to eliminate the possibility of high dilutions of broth supplying nutrient material, 1.0 c.c. of water from a flask which had been inoculated as usual and incubated was transferred to a fresh flask of distilled water with talc. This was incubated for two days and 0.1 c.c. was transferred to a third flask and flask-to-flask passage was continued in this way until the sixth flask was reached. The inoculum which it received was 300 per c.c.

and on the third day of incubation the count in it was 720,000 per c.c. The dilution of broth in this flask was approximated $1 : 25 \times 10^{21}$.

Experiment 7. The cotton wool used to plug the flasks must be considered as a possible source of carbon. This was eliminated by an experiment in which a flask was used with a beaker inverted over its neck and no cotton wool plug. An inoculum of 320 per c.c. increased to 960,000 per c.c. on the second day.

Experiment 8. This experiment was designed to eliminate at the same time the factors separately considered in the previous experiments.

The talc was prepared by roasting for several hours in a muffle furnace and by boiling for 72 hours in concentrated sulphuric acid. It was then again roasted for 48 hours in a muffle furnace. All the distilled water used was triply distilled, first from a metal still and then twice from a Pyrex all-glass still. For the second and third distillations potassium permanganate was added to the water undergoing treatment. All the glassware was most carefully cleaned, being finally repeatedly rinsed with distilled water. Flasks A to E were plugged with cotton wool; flask F was unplugged and was covered with an inverted beaker.

Flask A was inoculated with a dilution in distilled water of a repeatedly washed suspension of bact. E prepared from an agar slope. After four days' incubation of flask A approximately 0.25 c.c. of its contents was transferred with a capillary pipette to flask B. After a further four days' incubation flask C was similarly inoculated from flask B. This serial flask-to-flask transfer was continued, inoculation being done in each case on the fourth day.

The recorded counts in the flasks on the fourth day were as follows :—

A	520,000
B	740,000
C	680,000
D	600,000
E	480,000
F	600,000

Having thus eliminated the talc, the water, the inoculum and the cotton wool plug as sources of nitrogen and carbon, we are forced to the conclusion that the only source remaining is the atmosphere.

Experiments with different atmospheres

Many experiments were performed to investigate the effect of different atmospheres on the growth of bact. E in distilled water containing talc. The apparatus chiefly used was a thick-walled Pyrex flask fitted with a two-holed rubber stopper. Through one hole passed a straight glass tube and through the other a glass tube bent at a right angle. Both terminated in rubber tubes fitted with clips.

The necessary amounts of distilled water and talc were put into the flask and the stopper fitted. The water was boiled for five minutes, the steam being allowed to escape first through one tube and then through the other.

While steam was vigorously emerging, both clips were closed and the source of heat removed. On cooling, the steam inside the flask condensed and a vacuum was created. With stout Pyrex flasks the breakages were not considerable. When the temperature of the water had fallen almost to that of the air, a boiled thistle funnel was connected to the rubber tube at the end of the straight glass tube. The inoculum, diluted with freshly boiled and cooled distilled water, was introduced into the thistle funnel and, by cautiously opening the clip, it was forced into the flask. This was followed by 23 c c of boiled and cooled distilled water which washed any inoculum remaining in the tube into the flask. During these operations great care was taken to prevent the entry of air into the flask with the inoculum or the washing water. When the contents of the flask had attained room temperature, air, either untreated or treated, or other gases were admitted into the flask through the bent tube. The flask was then incubated and, after the selected period of culture, the stopper was removed and a sample taken for counting. In this method it was possible to count the number of bacteria present in a flask after incubation only once. The number of bacteria in the inoculum was ascertained by setting up a similar flask containing the same quantity of water and inoculum and sampling this.

Thirteen experiments were done with distilled water and talc inoculated with bact. E and incubated in air enclosed in the flask.

The sealed flasks used for these experiments (nominally 500 c c) had an actual capacity of about 550 c c. The volume of distilled water and inoculum was approximately 250 c c and the volume of air enclosed was therefore about 300 c c. In five experiments growth occurred, in eight it did not. In no case did bact. E fail to grow in distilled water *plus* talc when air was freely admitted. In these thirteen experiments, in which the amount of air available was limited, growth failed to occur eight times.

Experiment 9 The same amounts of distilled water (250 c c), talc (0.25 g) and inoculum (1 c c) were introduced into two sealed flasks, one of 550, the other of 1050 c c. The air available was in each case enclosed, and amounted in the first flask to 300 c c and in the second to 800. Counts were made after three days' incubation. Growth occurred in the flask containing 800 c c of air, not in the other.

Experiment 10 Five sealed flasks, each of 550 c c capacity, contained varying amounts of distilled water, talc and inoculum, but the proportionate amount of talc and inoculum to distilled water was constant (0.1 g of talc per 100 c c and 310 bact. E per c c). Counts were made after three days' incubation. Where the volume of fluid was 100 c c and the volume of air therefore 450 c c, growth occurred. Where, as a result of increasing the volume of fluid, the volume of air was less (350, 250, 150 and 50 c c), growth did not occur.

Experiment 11 For this and some subsequent experiments, the amount of enclosed air available was increased by connecting the bent tube of the flask to the corresponding tube of a similarly

fitted empty flask. In the single flask experiment, the volume of air available was 300 c.c.; in those with linked flasks, the volume available was 850 c.c. Flasks were opened for counting after various periods of incubation (table IV).

TABLE IV

The effect of the volume of air available on the growth of bact. E in distilled water and talc

Flask no.	Type of flask	Bacteria per c.c. inoculated	Bacteria per c.c. after incubation for (days).		
			2	4	6
1	Single	210	15
2	"	210	...	13	...
3	"	210	0
4	Linked	210	2
5	"	210	...	130,000	...
6	"	210	560,000

Three hundred c.c. of air did not provide sufficient essential materials but, in two cases out of three, 850 c.c. did.

For the next nine experiments, single flasks were used but air was admitted to them at a pressure less than that of the atmosphere. A pressure of two inches of mercury below that of the atmosphere was used on three occasions; on only one did growth occur. On no occasion did growth occur when the pressure was four or six inches of mercury below that of the atmosphere.

In these experiments the oxygen of the enclosed air was ample for even the most exacting aerobe. The nitrogen, if it was utilised, would have been amply sufficient. The results of the experiments suggest that some of the minor constituents of the air are required for the growth of bact. E in distilled water *plus* talc. When the amount of air is unlimited, sufficient of these constituents are always available to permit growth. Reduction of the amount of air below a minimum, which varies from day to day, prevents growth by reducing below the minimum the available amount of these minor constituents, the proportion of which in the atmosphere also varies from day to day.

Similar experiments were carried out using single flasks, linked flasks and jars incubated *in vacuo* and in the presence of oxygen, nitrogen and hydrogen. In no case did growth occur.

The experiments recorded appear to show that bact. E grows in distilled water and talc only when the atmosphere consists of air and when the amount of air available is considerable. The suggestion has already been made that neither the oxygen nor the nitrogen of the air acts as a nutrient and that the requisite food factors are some of the gases present in small amounts in the air.

The most probable of these are carbon dioxide and ammonia. To investigate this hypothesis, air was aspirated through a train of two soda lime towers, four wash bottles containing 20 per cent NaOH and four containing 20 per cent H_2SO_4 . It was collected and stored over water containing a small amount of NaOH.

Air so treated was used as the atmosphere in six experiments in single flasks and four in jars of 2000 cc capacity. In no case did growth occur. Such a result might be due to the addition to the air of inhibiting substances, but it is believed that this possibility was ruled out by the method used.

The reagents employed absorb from air both carbon dioxide and ammonia. They may also absorb other gases present in small amount in the air and so we are not entitled to say more than that bact. E does not grow in distilled water *plus* talc in the presence of air treated with reagents which absorb carbon dioxide and ammonia.

Complete proof of the role of these two gases would be the finding that growth occurred when they were added in suitable amount to air deprived of them by the treatment described. Such an experiment has been tried, but so far unsuccessfully. It is possible that these two gases are alone insufficient and that, while they supply the carbon and nitrogen required by the bacteria for their growth, the stimulus provided by some of the other minor constituents of the air, such as sulphur dioxide or hydrogen sulphide, is also required before the bacteria can commence to multiply.

*Growth in distilled water plus ammonium carbonate
without talc*

Carbon dioxide and ammonia combine with water to form ammonium carbonate. This suggested the possibility of the bacteria utilising this salt as nutrient material. Early experiments with ammonium carbonate in distilled water in concentrations ranging from 1 per cent to 1 per thousand gave negative results. When lower concentrations were used, however, growth occurred.

For these experiments a 1 per cent. solution of ammonium carbonate in distilled water was sealed in a glass tube and heated for half an-hour at $100^\circ C$. When cold the solution was diluted with cold boiled distilled water and the requisite amounts of the dilution were added to a series of flasks of cold boiled distilled water to give the concentrations used in the experiment. The flasks were inoculated with bact. E and incubated in air.

Seven experiments, each testing from three to seven different concentrations of ammonium carbonate, were put up. In six of these, at least one of the concentrations tested was found to give growth. The highest concentration in which growth occurred was 1.33×10^6 and the lowest 1.100×10^6 . Growth occurred on three

occasions in a concentration of $1:50 \times 10^6$. In view of the narrow range of concentrations favourable to growth, it is not surprising that growth of coliform bacilli in solutions of ammonium carbonate has not, so far as we are aware, been hitherto recorded.

The growth of bact. E in solutions of ammonium carbonate in distilled water supports the hypothesis which we advance that the growth of this bacterium in distilled water *plus* talc is due to the absorption by the water-talc mixture of sufficient carbon dioxide and ammonia from the air to act as nutritive materials. That the talc plays an essential part, probably by absorbing the gases, is shown by the fact that, in very many experiments, growth never occurred in distilled water which did not contain added substances, soluble or insoluble. It will suffice to quote one experiment.

Experiment 12. Five flasks, each containing 250 c.c. of distilled water, were autoclaved for 15, 11, 5, 2, and 0 days before they were inoculated each with 380 bact. E per c.c. The inoculated flasks were incubated and, in each case, the count after one day was zero. No increase occurred on further incubation.

Effect of varying the amount of talc

In most of the work done, the amount of talc used was 0.25 g. in 250 c.c. of distilled water. The effect of different concentrations of talc was investigated in an experiment in which the amount varied from 0.05 to 31.25 g. per 250 c.c. Growth occurred in all. In a further experiment growth occurred in the presence of 0.05 g. of talc but not when the amount was 0.01 g. or less.

From these experiments it may be concluded that the precise amount of talc present is comparatively unimportant provided that more than a minimum, which is probably between 0.01 and 0.05 g. per 250 c.c., is present. Since, however, with large amounts the peak is reached rather slowly, the amount empirically selected (0.25 g.) is probably best for investigations made to determine whether, under different conditions, talc-water mixtures give growth.

Effect of varying the total volume of fluid

Experiment 13. For routine investigation, 500 c.c. flasks containing 250 c.c. of distilled water with talc were employed. Growth can equally well be demonstrated with smaller amounts. For example, 10 c.c. of distilled water, autoclaved with 0.01 g. of talc in a test-tube, were inoculated with 0.5 c.c. of a $1:1,000,000$ dilution of a broth culture of bact. E and incubated. The initial count was 80 per c.c. On the fourth day the count was 800,000 per c.c.

Experiment 14. As a matter of convenience, in the majority of experiments the distilled water was boiled after the addition of

the talc That this was not essential is shown by an experiment in which talc sterilised by roasting was added when cool to distilled water which had been boiled and cooled and was not heated after the addition of the talc The peak recorded in water in which the talc had been boiled was 840,000 per c c , in the water to which talc had been added when the water was cool it was 1,300,000 per c c

Separation of talc and bacteria

The hypothesis has already been advanced that talc acts as an absorbent of gases from the air and renders them available for the growth of the organism This theory implies the necessity for the talc and the distilled water to be in intimate contact In a number of experiments distilled water containing talc was separated by a membrane from distilled water containing the inoculated bacteria The membranes used were, cellophane, parchment and sintered glass In none of these experiments did growth occur

Experiment 15 70 c c of distilled water containing 0.07 g of talc were introduced into the bulbs of two Buchner tubes which were autoclaved and then left standing upright overnight Next day, autoclaved and cooled distilled water was added to each In tube A care was taken not to disturb the deposited talc , the contents of B were mixed Both were inoculated with bact E and incubated Growth occurred with a peak of 1 600,000 per c c in B Growth did not occur in A

It appears legitimate to conclude from the results of these experiments that talc converts distilled water into a culture medium in the presence of air only when the talc is dispersed through the water If, as we suppose, it acts by adsorbing carbon dioxide and ammonia from the air, it appears probable that the bacteria grow in the immediate vicinity of the talc, where the nutritive materials are accumulated It is necessary to add, however, that microscopic observation of talc which had been present in distilled water in which bact E had grown, did not show any aggregation of bacteria around the particles

Several attempts were made to determine whether distilled water which had been in contact with talc acted as a culture medium when separated from the talc When the water was separated by filtering through Chamberland or sinter filters no growth occurred In some experiments in which separation was effected by centrifuging, growth occurred but in these cases the separation was incomplete To remove talc completely three centrifugings were found to be necessary, and when this was done growth did not occur in the distilled water which had been in contact with talc for periods varying from one hour to five days

Talc renders distilled water growth supporting only when it is

actually present. Distilled water which has been in contact with talc in no way differs, as regards its power of supporting the growth of coliform bacilli, from distilled water which has never been in contact with talc.

Experiments with filters

Chamberland filters, as the following experiments show, act on distilled water in much the same way as talc.

Experiment 16. A Chamberland filter was immersed in distilled water and autoclaved. After cooling the water was inoculated with 370 bact. E per c.c. and incubated with the filter still in position. On the third day the count was 140,000 per c.c.

Experiment 17. A new Chamberland filter was autoclaved in distilled water and was kept immersed in it. Another similar filter and a second sample of distilled water were autoclaved separately and when both were cold the filter was placed in the water. Both waters were inoculated and incubated, the filters remaining in the water throughout the period of incubation. In both cases growth occurred, the peaks being 2,500,000 per c.c. in the first sample and 1,600,000 in the second.

This experiment shows that it is not necessary for the filter to have been heated in the water to confer on it growth-supporting properties.

Experiment 18. Distilled water was autoclaved with a Chamberland filter. When cold, the filter was removed, drained and immersed in freshly autoclaved and cooled distilled water. Both waters were inoculated. Growth occurred in the water in which the filter was immersed but not in the water from which the filter had been removed.

In this experiment water in which a filter had been autoclaved and from which it was removed before inoculation was not growth-supporting, whereas water in which a filter was present during incubation was growth-supporting.

This experiment suffered from the defect that the period of contact of the water with the filter was short. In the next, longer periods of contact were allowed.

Experiment 19. Freshly roasted filters were autoclaved in distilled water, and, after autoclaving, were left in the water for various periods before removal. The waters were then inoculated with bact. E and incubated. No growth occurred in any sample.

This experiment shows that contact of distilled water with a porcelain filter in the presence of air for various periods does not impart growth-supporting properties to the water. Growth occurs only when the filter remains in the water after inoculation and during incubation. This finding is in agreement with that recorded for talc.

The effect of varying the inoculum

The inoculum normally used was 10 c.c. of a 1 : 100,000 dilution of a twenty-hour broth culture of bact. E to 240 c.c. of distilled water containing talc. This inoculum usually gave a count of 200-500 bacteria per c.c.

Experiment 20. In this experiment, the inocula were 1.0, 0.5 and 0.1 c.c. of a 1 : 100,000 dilution of a broth culture of bact. E, the standard amounts of distilled water and talc being used. The counts on inoculation were 50, 25 and 5 per c.c. respectively. Growth occurred in all cases and the size of the inoculum appeared to make very little difference to the peak counts attained on incubation.

Long period investigations

In the majority of the experiments the only information sought was whether, under a particular set of conditions, growth did or did not occur. Only three long period investigations of the rise and fall of counts were undertaken. In two of these the temperature of incubation was 37° C. and in one 22° C. In each case the flasks contained 250 c.c. of distilled water and 0.25 g. of talc.

In each case the counts showed peaks and troughs similar to, but less marked than, those recorded in natural waters (Bigger, 1937). The bacilli were still present in larger numbers than at the time of inoculation when the samples were exhausted, which occurred at 206 and 220 days at 37° C. and at 335 days in the experiment conducted at 22° C.

Experiments with natural waters

All the experiments so far recorded were carried out with distilled water. Tap water (from either the Ventry or Rathmines supplies of Dublin) was used with the usual quantity of talc in a number of experiments.

In only one case did growth occur, and this only to a small extent, in raw natural water containing talc. In every case growth occurred in autoclaved natural water containing talc, irrespective of whether the autoclaved water without talc supported growth. The results support the view advanced by Bigger that natural waters may contain both growth-supporting and growth-inhibiting substances. The inhibitory substances prevent the organism from utilising either the naturally occurring nutritive substances or the atmospheric gases rendered available by talc.

Experiments with other bacteria

In all the work so far recorded bact. E was the organism used. This has the same characteristics as a typical *Bact. coli* except that it is non-motile and fails to ferment ducitol.

Nineteen other coliform bacilli were tested in the usual way and every one was found to grow in talc and distilled water. The lowest peak count recorded was 110,000 per c.c. (inoculum 280 per c.c.). Five of the twenty organisms had been isolated from faeces and thirteen from urine; two were old stock cultures. Six of these organisms were typical *Bact. coli*, two were typical *Bact. aerogenes* and the rest intermediates. From this it may be concluded that the majority of coliform bacilli grow in talc and distilled water.

In addition to coliform bacilli, certain other organisms were tested. Two strains of *Bact. pneumoniae* yielded peaks of 500,000 and 300,000 per c.c. (inocula 360 and 480 per c.c. respectively). One strain of *Bact. alcaligenes* gave a peak of 680,000 per c.c. from an inoculum of 180 per c.c. Neither *Proteus vulgaris* nor *Staphylococcus pyogenes* grew.

A more extensive testing of different bacterial species was not undertaken. Sufficient work has been done to show that the power of growing in distilled water *plus* talc is widely distributed among various types of Gram-negative bacilli.

Experiments with other insoluble inorganic substances

The next question which presented itself was whether talc was the only inorganic substance capable of rendering distilled water growth-supporting. Seventy-five insoluble or almost insoluble inorganic substances were tested by suspending 0.25 g. in powder form in 250 c.c. of distilled water. After boiling or autoclaving and cooling, the fluid was inoculated as usual with bact. E and incubated. Counts were made at intervals of one or two days until either a satisfactorily high level or zero had been attained. The lowest peak count among the substances promoting growth was 120,000 per c.c. from an inoculum of 320 per c.c.

The following twenty substances in addition to talc were found to render water growth-supporting :—

ASBESTOS	MAGNESIUM SILICATE
BARIUM HYPOSULPHITE	MANGANESE DIOXIDE
BARIUM SULPHIDE	PERMUTIT
BARIUM SULPHITE	SILICA
CALCIUM HYPOSULPHITE	SILVER SAND
CALCIUM PHOSPHATE	SOIL
CALCIUM SULPHATE	UNGLAZED PORCELAIN (CHAMBERLAND
FERROUS PHOSPHATE	FILTER)
FERROUS SILICATE	UNGLAZED PORCELAIN (POROUS POT)
KAOLIN	ZIRCONIUM SILICATE
KIESELGUHR	

It seems to be quite impossible to find any common factor in this highly heterogeneous group of substances. We may say, however, without fear of contradiction, that a large number of insoluble or almost insoluble inorganic substances, when added to distilled water, render it capable of supporting the growth of coliform bacilli.

A certain number of organic substances have been tested in a similar manner but it is not proposed to present the results here.

DISCUSSION

This research commenced with an attempt to determine whether Harold or Bigger was correct as regards the effect of candle filtration on natural and sand filtered waters. It has clearly demonstrated that certain waters which, in the untreated state, do not permit the growth of coliform bacilli, are rendered growth-supporting by filtration through Chamberland filters and that this effect is produced even when all contact with rubber is avoided. This confirms Bigger's hypothesis that filtration removes inhibitory substances and so permits the growth of coliform bacilli which utilise the nutritive materials present in the water.

The research developed in a way which, when it was initiated, was quite unforeseen. Harold's observation that distilled water, by being boiled in the presence of rubber tubing, was rendered capable of supporting the growth of coliform bacilli was confirmed and it was proved that this was due to the presence in the water of talc, used as a surface dressing for the rubber. Later it was shown that this power was not peculiar to talc but was shared by twenty other insoluble inorganic substances, some naturally occurring and others pure chemical compounds.

The twenty coliform bacilli examined, including typical *Bact coli*, typical *Bact aerogenes* and various intermediates, grew in distilled water plus talc.

The experiments recorded proved that nutrient material was neither present as an impurity in the distilled water or talc, nor introduced with the inoculum or from the cotton wool plugs. That carbon and nitrogen, elements essential for the growth of the bacteria, must have been derived from the air is established by the exclusion of other sources and the absence of growth *in vacuo* and in other gases. The hypothesis is advanced that the sources of the essential elements are the carbon dioxide and ammonia of the atmosphere. This hypothesis is supported by the experiments which showed that not merely air, but air in large amount, is necessary for the growth of coliform bacilli in distilled water talc preparations, that growth does not occur in air deprived of carbon dioxide and ammonia and that growth does occur in distilled water containing ammonium carbonate in high dilution but no talc. It is

not claimed that complete proof of the hypothesis has been presented, as experiments designed for this purpose have not yet been successful. Despite this, there is a strong presumption that the hypothesis is true. The hypothesis does not exclude the possibility that other minor gaseous constituents of the atmosphere may also be required.

The role of the talc is obscure. That it or one of the other insoluble substances plays an essential part is shown by the invariable growth of coliform bacilli in distilled water *plus* talc in the presence of unlimited air and their invariable failure to grow in distilled water without talc. Talc does not impart growth-supporting properties to distilled water with which it has been in contact and from which it has been separated; it acts only when present in the water inoculated with the bacteria. We probably cannot do better than assume that talc acts as a catalyst, rendering the carbon dioxide and ammonia of the air (if indeed, as we believe, these are the essential food materials) available for the growth of the bacteria. In the phenomena here described the insoluble substances appear to play a more important part than do these or other insoluble substances when they act as adjuvants to the growth of *Cl. tetani* in injured tissues or *Mycobacterium tuberculosis* in the silicotic lung.

The coliform bacilli have long been regarded as facultatively pathogenic saprophytes. Our findings that they can grow in distilled water containing insoluble inorganic substances, including soil, and can utilise as food such simple chemical substance as ammonium carbonate, entitle them to be considered also as facultatively autotrophic. The correctness of this classification is confirmed by their growth in Winogradsky's medium, the commonly accepted criterion by which an organism is judged to be an autotroph.

Presuming the correctness of our hypothesis, it is of interest to consider how the organisms growing in distilled water-talc preparations obtain the energy necessary for their metabolism. Normally coliform bacilli obtain energy by the oxidation of carbon-containing substances. Carbon dioxide is not further oxidisable and in our experiments light was excluded, so energy could not have been obtained from either of these sources. There remains the nitrogen-containing substance, and it appears probable that it is by the oxidation of ammonia that the bacilli obtain the energy which they require for their metabolism.

It may well be asked if the phenomenon of growth of coliform bacilli in water containing an insoluble inorganic substance is of more than theoretical interest. Does this type of growth occur under natural conditions and, if so, is it likely to render unreliable the commonly used methods of assessing the hygienic quality of water? So far as our experiments have gone, they tend to give a negative answer to the first of these questions. When we substitute natural for distilled water in a water-talc preparation, we introduce

inhibitory substances These are usually capable of preventing the growth of coliform bacilli whether the food material is that produced by the action of talc on atmospheric gases or that present in the majority of natural waters Under certain conditions, however, the inhibitory substances may be present in insufficient amount and then growth may result from the presence of either type of nutrient or a combination of both Among the conditions favouring the destruction of inhibitory substances heat is the most important and we believe that, in water subjected to a temperature likely to be encountered in tropical countries, growth of coliform bacilli might occur to an extent sufficient to invalidate conclusions based on bacteriological examination It is intriguing to find sand and permutit, which are used for the purification and softening of water, among the substances imparting to water growth supporting properties but it is doubtful if, in actual use either of these would lead to a multiplication of coliform bacilli

SUMMARY

1 We confirm Bigger's conclusion that filtration of natural waters which in their untreated state, are not capable of supporting the growth of coliform bacilli may render them growth supporting Contact with rubber is not required to effect this change

2 We confirm Harold's finding that distilled water may be rendered growth supporting by contact with rubber tubing

3 The substance responsible for this effect is talc, used as a surface dressing for rubber

4 A coliform bacillus, bact E, never failed to grow in plugged flasks containing distilled water and talc (0.25 g per 250 c.c.)

5 Although the addition of talc to distilled water converts it into a culture medium, the talc does not itself supply the necessary nutritive materials

6 Growth in distilled water-talc preparations is not due to impurities in the talc or distilled water or to organic material introduced with the inoculum or cotton wool plugs

7 Growth does not occur in distilled water talc preparations *in vacuo*, nor in oxygen, hydrogen or nitrogen

8 The necessary nutritive materials are derived from the atmosphere

9 Growth does not occur when the amount of air available is restricted or when the air has been treated with soda lime, sodium hydrate and sulphuric acid

10 The nutritive materials are some of the minor gaseous constituents of the atmosphere The hypothesis is advanced that these are carbon dioxide and ammonia, although it is possible that other gases may also be necessary

11. Bact. E grows in solutions of ammonium carbonate in distilled water, provided the concentration of the salt is suitable.

12. Coliform bacilli are facultatively autotrophic.

13. To render distilled water growth-supporting, talc must be present in and, to some extent, dispersed through the water. Distilled water which had been in contact with talc and had been completely separated from it was not growth-supporting.

14. In distilled water-talc preparations, alternation of high and low counts of coliform bacilli occurs. The organisms survive for long periods—220 days at 37° C. and 335 days at 22° C.

15. The addition of talc to tap water which had been heated invariably made it growth-supporting, even when the heated water without talc was not, but the addition of talc to raw tap water did not make it growth-supporting. This is believed to be due to the presence in tap water of heat-labile inhibitory substances.

16. Each of twenty coliform bacilli tested grew in distilled water-talc preparations. These included typical *Bact. coli*, typical *Bact. aerogenes* and various intermediates. *Bact. pneumoniæ* and *Bact. alcaligenes* also grew in these preparations.

17. Twenty other insoluble inorganic substances out of 75 tested acted like talc in rendering distilled water growth-supporting.

We express our thanks to the Department of Education and the Medical Research Council of Ireland for the grants made to the junior author. We are most grateful to Dr R. A. O'Meara, Professor E. J. Conway and Captain Ramsden for their assistance and advice. Without the willing help of the staff of the School of Pathology the work recorded here could not have been accomplished.

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MALIGNANT HYPERTENSION ASSOCIATED WITH HYDATID DISEASE OF THE KIDNEY

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(PLATE XVI)

IN about 2 per cent. of cases of hydatid disease in man cysts occur in the kidney (Dew, 1928; Grindlay and Walters, 1940). The condition is therefore rare, but the present case is reported because malignant hypertension was also present and it seemed probable that the mechanism of its production was similar to that by which hypertension has been produced experimentally by Wilson and Pickering (1937-38), Goldblatt (1938) and Wilson and Byrom (1939), *i.e.* by constriction of one or both renal arteries.

Since the publication of Goldblatt's important experiments, a considerable number of cases have been reported of hypertension associated with renal disease in which the blood supply of one or both kidneys had suffered restriction and in which the suggestion was made that a mechanism analogous to that of the experimental technique had operated in the production of the hypertension. The bulk of these cases have been recently reviewed by Patch *et al.* (1940), Palmer *et al.* (1940), Perry and Taylor (1940) and McCann (1941). Furthermore, quite large series of cases of non-nephritic renal disease have been the subject of statistical enquiry to determine whether or not there is a significant correlation between these diseases and hypertension. A definite correlation was claimed by Maher and Wosika (1939), Cohn *et al.* (1939), Weiss and Parker (1939) and Palmer *et al.* (1940), while Morlock and Horton (1936), Crabtree (1940), Pearman *et al.* (1940) and Braasch *et al.* (1940) found little evidence in favour of an aetiological relationship.

The present case provides a further instance in which the operation of the Goldblatt mechanism of the production of hypertension may be postulated in the human subject.

Case history

A young married woman, aged 25, was admitted into the Manchester Royal Infirmary under Dr Bramwell, 24.8.40, complaining of headache, vomiting, cough, weakness and lassitude of about two years' duration. Her

symptoms started with migraine-like attacks two or three times a week after waking in the morning. The headache was usually unilateral and the attacks, which were often relieved by vomiting, were associated with great lassitude. Ten months before admission she miscarried at three months, and for some months before admission she suffered from night sweats. Her blood pressure was first taken about 3 weeks before admission and was then 220 mm. Hg systolic, the diastolic pressure not having been recorded. Swelling of the feet and ankles had been noticed after standing.

On admission the heart was found to be enlarged, the apex beat being palpable external to the mid-clavicular line. Her blood pressure was now 200/100 mm. Hg. No masses were palpable in the abdomen. There was oedema of the sacral region. The discs were pale. The urine contained 2 mg. of protein per litre (Esbach). The blood urea was 196 mg. per 100 c.c. She died ten days after admission with a blood urea of 378 mg. The clinical diagnosis was chronic nephritis.

Post-mortem findings

Lungs showed intense oedema and congestion. *Heart* (450 g.) was enlarged and exhibited typical "concentric" hypertrophy of the left ventricle. There were no valvular lesions. *Liver* showed patchy fatty change. *Spleen*, brick-red in colour, was fairly soft.

Right kidney. The lower pole was occupied by a cystic swelling measuring 6×5 cm. On section this contained white caseous material intermingled with flat masses of soft greenish semi-translucent material. The cystic mass appeared separate from the pelvis and was enclosed by a fibrous capsule. The residual renal substance measured 8 mm. at its thickest part and about 3 mm. where it had been most encroached upon by the cyst. The pelvis and calyces were considerably dilated, but there was no obvious communication with the cyst cavity. After preliminary fixation it became evident that the flat translucent masses had formed a lining to the cyst and direct microscopical examination of the contents showed the presence of numbers of necrotic scolices and very many hooklets, indicating the diagnosis of hydatid disease. In addition there was considerable polymorphonuclear cell infiltration.

Left kidney. This was somewhat enlarged, weighing 200 g. The capsule stripped easily, exposing a finely granular surface mottled purplish yellow. On section the cortex was mottled dark red and pink and the pattern was rather indistinct. Demarcation from the medulla was clear. There was no abnormality in either pelvis or ureter.

Histological examination

Right kidney. The appearances were similar to those seen in a long-standing hydronephrosis which had produced compression of the renal substance. Very many of the glomeruli were completely fibrotic and there was irregular fibrosis throughout the parenchyma.

MALIGNANT HYPERTENSION AND RENAL HYDATID



FIG. 1—Left kidney, showing fibrotic branch of renal artery adjacent to wall of hydatid cyst. Weigert's elastin stain and van Gieson $\times 30$



FIG. 2—Right kidney. Afferent arteriole exhibiting necrotizing arteritis. Glomerulus showing focal necrosis. H and E $\times 80$



FIG. 3—Right kidney. Glomerulus and afferent arteriole showing typical fibrinoid necrosis. Mallory's stain $\times 200$



FIG. 4—Right kidney. Glomerulus showing hyaline changes in tuft. H and E $\times 200$

The cyst was enclosed within a capsule of dense fibrous tissue, while the wall of the cyst itself was composed of hyaline clutinous substance, with many related necrotic scolices. In the renal substance close to the cyst wall could be seen several fairly large branches of the renal artery showing fibrosis of the media and partial or complete fibrous obliteration of the lumen (fig 1). Smaller branches of the renal artery within the surviving renal parenchyma showed variable degrees of intimal fibrosis. There were no signs of necrotising arteritis nor of focal glomerular necrosis.

Left kidney There appeared to be little diminution in the number of glomeruli, but few of them were normal. The outstanding glomerular lesion was focal necrosis (figs 2 and 3), which affected about a quarter of the whole number. In addition many showed variable degrees of hyalinisation (fig 4) and a few were completely fibrosed. A number of glomeruli exhibited capsulitis and some were the seat of hæmorrhagic infarction. The tubules generally showed degeneration, groups of convoluted tubules were crammed with red blood corpuscles and many others contained hyaline casts. Of the vessels, the afferent arterioles showed necrotising arteritis, often the whole thickness of the arteriole exhibited fibrinoid necrosis giving the characteristic fuchsinophile staining with Mallory's stain (fig 3). Other afferent arterioles showed degrees of intimal fibrosis up to complete obliteration of the lumen. Many of the intralobular arteries showed considerable intimal fibrosis (endarteritis fibrosa). The larger branches of the renal artery within the substance of the kidney showed no pathological change, contrasting with the larger branches of the right renal artery adjacent to the cyst wall, which showed considerable fibrotic stenosis. There was a diffuse, slight to moderate increase in fibrous tissue throughout both cortex and medulla, most evident in the neighbourhood of the few completely fibrosed glomeruli.

Summary of renal lesions

Right kidney Suppuration within a hydatid cyst. Dilatation of renal pelvis with gross compression of renal substance. Partial obliteration of moderately large branches of the renal artery. Fibrosis of parenchyma. Absence of typical histological lesions of malignant hypertension.

Left kidney Characteristic histological picture of malignant hypertension (Kimmelstiel and Wilson, 1936, Ellis, 1938, Wilson and Byrom, 1939).

Discussion

The first question to be decided in this case is whether the malignant hypertension developed as the result of the presence of the hydatid cyst or whether it was merely the terminal phase of a

pre-existing chronic nephritis. The clinical history indicates that symptoms had been present for at least two years. If a pre-existing chronic nephritis had been responsible for these symptoms a greater number of completely fibrosed glomeruli and a considerable degree of disorganisation of the renal architecture would have been found in the left kidney. On histological grounds therefore it is possible to exclude a pre-existing chronic nephritis as the ætiological agent in the production of the malignant hypertension. The presence of the many fibrotic glomeruli in the right kidney is explicable as the result of compression by the cyst of the blood vessels of the kidney or as secondary to the hydronephrosis.

Malignant hypertension is rare in young adults and, pre-existing chronic nephritis having been eliminated, it is legitimate to regard the presence of the hydatid cyst as the ætiological factor in this case. Such cysts grow slowly, usually over many years (Dew, 1928), and it may reasonably be inferred that the cyst was of a size to interfere in some measure with the renal circulation for a considerable period before the onset of symptoms. The fact, too, that typical lesions of malignant hypertension were not seen in the right kidney at least indicates that the constriction of its vessels occurred before the development of the hypertension. Chronologically, therefore, there is no objection to regarding the presence of the hydatid cyst as the ætiological factor.

The site, encroaching on the pelvis as well as on the parenchyma of the kidney, was such as to make it fairly certain that the cyst had brought about some degree of compression of the main renal vessels, and histologically it is evident that some of the larger branches within the renal substance adjacent to the cyst wall showed advanced fibrotic diminution of the lumen.

It has been shown experimentally (Wilson and Pickering, 1937-38; Goldblatt, 1938; Wilson and Byrom, 1939) that constriction of one renal artery will produce persistent hypertension with development of the characteristic lesions in the opposite kidney but not in the constricted organ. This case appears to furnish a close analogy with these experimental findings. A unilateral constricting factor, the hydatid cyst, is first present, and subsequently the typical lesions of malignant hypertension develop in the contralateral kidney but not in the constricted organ. Why the presence of a hydatid cyst in this case should lead to constriction producing hypertension and not in other cases may well depend on accidental factors such as site, rate of growth, etc. Three recent reports of hydatid disease of the kidney (Fisher, 1940; Barrett, 1940; Grindlay and Walters, 1940) make no mention of the occurrence of malignant hypertension. In Fisher's case the blood pressure is not even referred to, in Barrett's case the blood pressure was not raised, but in Grindlay's case the pressure was 150/100 mm. Hg.

However, as the patient's age was 50 years, no deductions can be drawn. In all three cases operative removal was successful, so that there were no histological data concerning the state of the contralateral kidney.

As already mentioned, attempts to show by statistical methods that a causal relationship exists between non-nephritic renal disease and hypertension have produced conflicting results. However, the studies by Oppenheimer *et al.* (1939) of 105 cases of unilateral renal disease tend to explain why hypertension does not necessarily follow in these cases. He found a statistical correlation of unilateral renal disease with hypertension only in those cases in which a unilateral narrowing of the renal arteries was present. His conclusions fall into line with the results obtained experimentally and help to explain the conflicting results obtained by statistical analysis when no account is taken of the exact anatomical conditions in each individual case. Again, careful anatomical investigations by Blackman (1939) showed that in 50 cases of essential hypertension coming to autopsy there was much narrowing of the lumen of one or both main renal arteries in 27 cases, and moderate narrowing in 16. This permitted him to infer that the Goldblatt mechanism had operated in the production of hypertension. Similarly Saphir and Ballinger (1940) described two patients with malignant hypertension secondary to unilateral vascular stenosis with consequent renal ischaemia.

Thus it may be expected that individual cases such as the one here described will continue to be reported as evincing evidence of the operation of the Goldblatt mechanism in the human subject, while mass investigations designed to show a statistical correlation between unilateral renal disease and hypertension will continue to yield equivocal results unless each case is investigated anatomically to determine the presence or absence of narrowing of the renal arteries.

Summary

1. A case of hydatid disease involving the left kidney is described.
2. In the right kidney the histological changes characteristic of malignant hypertension were present, but these were not present in the left kidney.
3. It is suggested that the case provides an example of the operation in the human subject of the Goldblatt mechanism of the production of hypertension.

I wish to thank Dr Crighton Bramwell for permission to publish this case and for kind assistance with clinical aspects, Professor S. L. Baker for helpful criticism and Mr H. C. Taylor for the photographs.

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THE EFFECT OF PRONTOSIL THERAPY ON THE ANTISTREPTOLYSIN O TITRE IN RABBITS DURING IMMUNISATION

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THE mode of action of prontosil and allied drugs has been the subject of intensive research, but there are few records of the effect of such therapy on antibody response, upon which the ultimate success of the treatment partly depends. Although much remains obscure, the general conclusion has now been reached that drugs of this group act mainly by producing bacteriostasis thereby permitting the natural defences to come into full operation. Without adequate experimental evidence, the suggestion has frequently been made that actual stimulation of these defences by the drugs or their derivatives may also be a factor.

In hæmolytic streptococcal infection Colebrook *et al* (1936) demonstrated that, although the addition of prontosil itself to the blood was without effect on bactericidal action, *after administration of this drug to animal and man*, the bactericidal power of the blood for small numbers of organisms was increased. This action was considered to be due to the reduction of the inactive prontosil to *p*-aminobenzenesulphonamide, which had feeble bactericidal powers. These findings have been confirmed but the possibility of some additional mechanism has been suggested to explain the apparently good therapeutic effect of the drugs. Exploring the possibility of specific tissue stimulation by prontosil, Gay and Clark (1937) could find no evidence, in experimental streptococcal infection in rabbits that the cell reaction which finally accounted for the disposal of the organisms was other than local, and concluded that the spread of infection was prevented by bacteriostasis. Levaditi and Vaisman (1935) recorded *in vitro* inhibition of streptococcal hæmolysin by prontosil I (4 sulphamido 2,4 diamino azobenzol) and concluded that certain sulphonamide compounds exerted an antitoxic action. Osgood (1938) also suggested that the drugs of this group inactivated hæmolysin and perhaps other toxic products. Gross *et al* (1938) found that prontosil II produced more marked inhibition of streptococcal hæmolysin *in vitro* than did sulphonamide, which had only a slight reaction. They also observed that the administration of sulphonamide *in vivo* and its addition to serum did not enhance the inhibitory effect of rabbit serum on streptococcal hæmolysin. On the other hand, Huntington (1938) concluded that the slight delay of hæmolysin production caused by sulphonamide in broth cultures was possibly due to modification of the growth curve. Sulphanilamide was without effect upon the formation of erythrogenic toxin.

in vitro and was unable to inactivate small amounts of the toxin when used in a concentration equal to or greater than that induced in body fluids therapeutically.

In this paper observations on the effect of prontosil therapy on the antistreptolysin O response of rabbits are recorded. This antibody can be accurately titrated and its production as part of the natural response to hæmolytic streptococcal infection has been fully demonstrated by Todd (1932a).

METHODS

Source of streptolysin O. The method described by Todd (1932b) for the production of streptolysin O was followed, the strain of hæmolytic streptococcus used being "Richards" (Lancefield group A, Griffith type 3). The same batch of streptolysin was used throughout, the titre being 8.4 units per c.c.

Antistreptolysin O (A.S.O.) titration. The method described by Coburn and Pauli (1935) was used.

Prontosil therapy. The drug used was prontosil II, the disodium salt of 4-sulphamidophenyl-2-azo-7-acetyl-amino-1-hydroxynaphthalene-3.6-disulphonic acid. Each injection of 2 c.c. of a 5 per cent. solution was given on the left side, half of it subcutaneously, half intramuscularly.

Implantation of agar foci. Ten c.c. of an 18-hour broth culture was centrifuged and the deposited organisms were suspended in 4 c.c. of melted agar at 40° C. Two c.c. of the suspension were immediately injected subcutaneously into the right side. The doughy mass of the inoculum persisted for several days and the site later became indurated. The regional glands were distinctly palpable for varying periods, but no fluctuation was detected and the overlying skin was not involved.

RESULTS

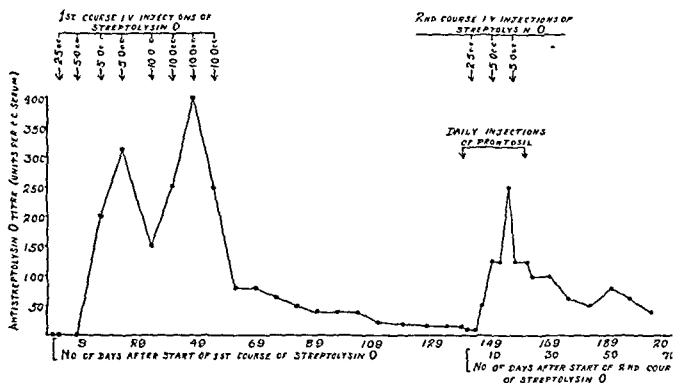
The effect of prontosil therapy on the antistreptolysin response to injections of streptolysin O

Two rabbits (12 and 13), weighing 3010 and 3420 g. respectively, were each given the same preliminary course of intravenous injections of streptolysin O as shown in fig. 1, venous blood being withdrawn for antistreptolysin O (A.S.O.) titration immediately before each injection. At the beginning of the experiment, the titres of both animals were below 1 unit per c.c. The titre of rabbit 12 had risen to 200 units on the 15th day whereas that of no. 13 was still less than 1. By the 22nd day, rabbit 13 had also responded, the titre being 125 units. The subsequent titre curves were almost identical in shape, but that of rabbit 12 was on a higher level throughout the course of injections. Peculiar similarities in the curves were noted. Thus a fall in titre after the 4th injection, given on the 22nd day, occurred in both animals. In both animals the titre reached its maximum level towards the end of the course but a sharp depression followed the seventh and eighth injections in rabbit 12, and the eighth injection in no. 13. After the initial

drop on cessation of the injections, the titre gradually fell. Rabbit 12 reached a basal titre of 16 units on the 125th day while that of no. 13 fluctuated between 6 and 10 from the 109th day.

As compared with rabbit 13, the response in no. 12 was more

RABBIT NO 12



RABBIT NO 13

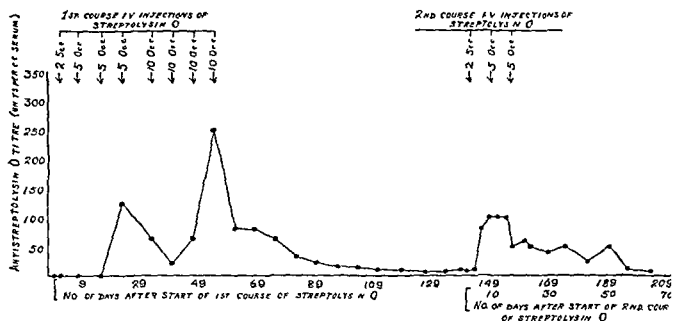


FIG. 1.—To show the effect of prontosil therapy on the antistreptolysin O response of rabbits following the injection of streptolysin O

rapid and was maintained at a higher level, but it reached a steady resting titre in approximately the same time.

When the titre had remained steady for some three weeks, the effect of prontosil therapy on the response to a second series of streptolysin injections was tested. Only three injections were

given and they were the same as the first three of the first course. As it seemed probable that any failure in response would be most manifest in the animal which had shown the greater primary reaction, rabbit 12 was used for the test and no. 13 retained as the control. Rabbit 12 was given 2 c.c. of a 5 per cent. solution of prontosil II daily from the 137th to the 158th day—44 c.c. in 22 days. Rabbit 13 received no prontosil. The A.S.O. titrations were performed at shorter intervals in order to detect the earliest effect. In both animals the titre rose on the 6th day after the second series of injections was begun. The titre curves were similar save that rabbit 12 was again at a higher level and reached a definite peak of 250 units, whereas the curve of no. 13 was flattened at the maximum level of 100 units (fig. 1).

There was no indication, therefore, that the antistreptolysin response was affected by the administration of prontosil. Nor was there any evidence of the in-vivo production, under prontosil therapy alone, of any substance capable of neutralising streptolysin O. for the titre of rabbit 12 showed a slight decrease rather than an increase after 48 hours' therapy (139th day).

The fact that in both animals the A.S.O. titre was below 1 unit at the commencement of the experiment indicated that streptolysin O was a completely new antigen to these animals. This was confirmed by the long latent period of 9 days or more before the primary response was obtained. The latent period preceding the rise in titre in the second part of the experiment was shortened to 4-6 days, this reduction being characteristic of a secondary response.

The effect of prontosil therapy on the antistreptolysin O response to experimental infection with hæmolytic streptococci

In four rabbits (14-17) of approximately equal weight, a preliminary A.S.O. titration revealed less than 1 unit per c.c. In the first part of the experiment, rabbits 14 and 15 were each given daily injections of 2 c.c. of a 5 per cent. solution of prontosil II for 23 days, 46 c.c. in all. After 72 hours' therapy, a focus of hæmolytic streptococci ("Richards") in agar was implanted in the right flank of both animals. The control rabbits, 16 and 17, received no prontosil but identical streptococcal foci were implanted. The streptococcal injections were repeated 24 hours later in all rabbits to ensure that infection occurred.

Rabbit 16 (untreated) appeared ill on the third day after infection and was found dead of hæmolytic streptococcal septicæmia the following morning. Rabbit 17 (untreated) was in poor condition from the 4th to the 11th day, but recovered. The A.S.O. curve is illustrated in fig. 2. This antibody was first detected on the 11th day, when the titre was 10 units. The titre reached a maximum of 80 units in 19 days and did not fall below 40 during the first 101 days,

save for an exceptional result on the 44th day when no trace of antibody was detected. This was the only occasion on which complete absence of antibody was observed in any animal in

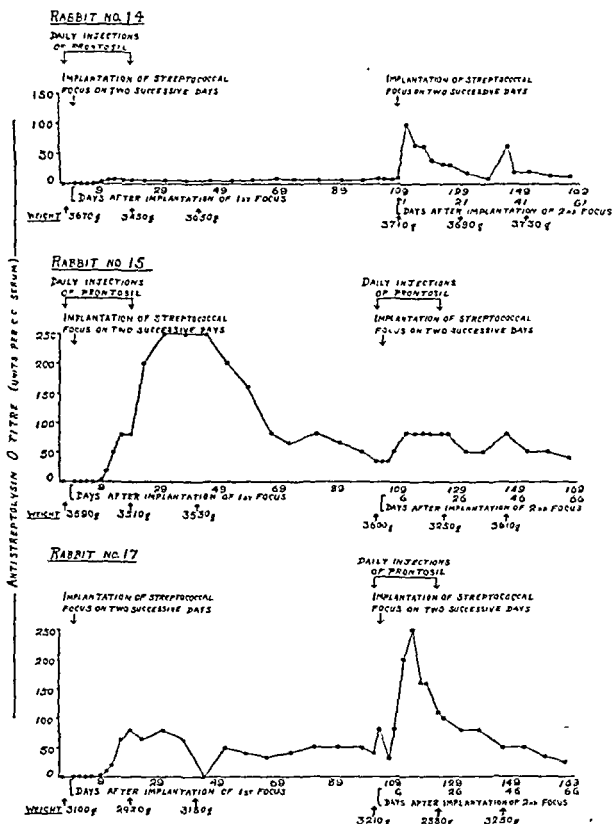


FIG. 2.—To show the effect of prontosil therapy on the antistreptolysin O response of rabbits following the implantation of foci of *Streptococcus haemolyticus*.

which it had once appeared. No explanation was forthcoming and repeated tests gave the same result.

Rabbit 14 (treated) did not appear to be in good condition from

the 4th to the 7th day but it quickly recovered, yielding the A.S.O. titre curve shown in fig. 2. The titre remained below 1 unit per c.c. until the 9th day, when it rose to 1 unit. A maximum titre of 8 units was reached in 11 days, and fluctuation between 6 and 8 units continued for over 100 days with an exceptional reading of 10 units on the 67th day. Rabbit 15 (treated) lost 440 g. between the start of the experiment and the end of the course of prontosil injections. The A.S.O. titre remained at zero until the 9th day, when it was found to be 3 units per c.c. It climbed rapidly to a maximum of 250 on the 29th day and maintained this level for 2-3 weeks. It then fell, at first rapidly but later more slowly, reaching 32 on the 101st day.

The virulence of the "Richards" strain was not tested but it was an effective pathogen as evidenced by the fate of rabbit 16 and the untoward effect on the other animals, particularly no. 17. As indicated in fig. 2, rabbits 14 and 15 had lost 220 and 440 g. respectively when the prontosil injections were terminated after 23 days, and this loss had been largely made good in a further 23 days. It seemed that part of this loss was due to the therapy.

So far as the titration curves were concerned, the control rabbit (17) retained a relatively high titre for a long period considering that the maximum titre was only 80 units. The curves of the test rabbits, 14 and 15, differed very considerably and illustrated the variation in individual response following identical treatment and dosage. Had selection from a larger number of tests been possible, it would have been difficult to produce greater extremes in reaction. A feature common to both was the series of zero readings while under prontosil therapy at the start of the experiment. This confirmed the previous observation that the exhibition of prontosil alone did not result in the in-vivo production of any substance capable of neutralising streptolysin O. On the other hand, prontosil did not delay the formation of antistreptolysin O, for this was present at an earlier stage in treated animals than in the untreated control. Nor did prontosil appear to limit the production of the antibody, since the response in one treated rabbit greatly exceeded that in the control.

In the second part of the experiment, the agar foci implanted in all three animals were identical with those of the first part, but rabbits 15 and 17 received prontosil and 14 did not. Prontosil was withheld from this rabbit in order to see if the continuously low A.S.O. titre had been due to inhibition by the drug. The sharp rise in titre of no. 15 on the 23rd day, when prontosil was stopped, together with the delay in reaching the maximum titre as compared with the control (17), also supported the hypothesis of inhibition. In this animal it was considered advisable to see if the

result could be repeated and prontosil was therefore given as before. Rabbit 17, which had previously served as control and received no prontosil, was now given a course of the drug.

Fig. 2 indicates that, three days after the implantation of the focus, rabbit 14 reached the relatively high titre of 100, which fell quickly to 10 on the 29th day. There next appeared an unexplained rise on the 37th day to 63, followed by a fall to 16 on the 39th day. The contrast between the first and second response suggested that there may have been some truth in the hypothesis that prontosil inhibited antibody production.

Forty eight hours' therapy did not alter the titre of rabbit 15 (103rd day). A rise to 50 units was first noted five days after the implantation of the streptococcal focus, and on the 8th day 80 units was reached. This level was maintained until the 28th day, when 50 units was recorded. Apart from a reading of 80 units on the 42nd day, the titre subsequently remained steady at 50 units. As the latent period before the secondary response was comparably shortened in both 14 and 15, the use of prontosil in the latter did not delay antibody production. Otherwise the secondary response in 15 was much less intense than the primary and indicated that quantitative variations alone must be cautiously considered before being accepted as significant evidence of therapeutic interference. Nevertheless the response was as great as the first reaction in 17 and the second in 14, in both of which prontosil was omitted.

In rabbit 17, the titre rose from 50 units to 80 after 2 days' prontosil therapy. Under the conditions of the test this rise was significant but no explanation could be found. The increase was not maintained under continued therapy, the titre being 32 units after 5 days' treatment, which indicated that the drug was not the likely cause of the transitory increase. After the implantation of the agar focus, the secondary response was manifested in a rise of titre to 80 units on the 5th day, followed by a further rise to a peak of 250 units on the 11th day. The titre then fell gradually to 50 units on the 42nd day. Despite prontosil therapy, the usual reduction in latent interval occurred before the appearance of the response and the response was greater than in the original course. This combined result was considered positive evidence of the absence of any interference with antistreptolysin O production by prontosil.

It may be noted that the test rabbits 15 and 17 lost 350 and 330 g respectively in the second part of this experiment. At the same time the untreated control (14) lost 220 g as a result of infection alone. It may be significant that the greatest loss in weight was associated in both parts of the experiment with the use of prontosil.

DISCUSSION

Despite the individual variation in response the results indicated little if any interference with antistreptolysin O production in experimental animals as a result of the simultaneous injection of hæmolytic streptococci and prontosil. It is to be expected that similar results would be found if other antibody reactions were investigated.

Apart from the academic interest of the subject, the problem is of practical importance in view of the wide application of prontosil therapy. The use of the drug is often followed by periods in which a focus of infection persists. It is important to ascertain, therefore, if there is any check to the development of the natural defence mechanism as a result of treatment with prontosil. So far as dosage was concerned, the daily injection in these experiments approximated to 0.03 g. of prontosil II per kg. body weight, the total amount injected over a period of 22-23 days being 0.66-0.69 g. Neglecting species differences, the equivalent amounts for a 70 kg. man would be a daily dose of 2 g. and a total of 64-66 g. Larger doses are commonly used in the early days of treatment to secure a maximum effect rapidly, but there was no necessity for this procedure in the experimental work, in which it was possible to institute therapy before infection occurred. Experimentally the drug was used at least 48 hours before infection so that any possible interference with antibody production was favoured as compared with human infections, in which the antigenic stimulus has been applied, in most cases, before therapy is commenced. It is a reasonable assumption that prontosil has no appreciable antagonistic effect on antibody production in human infections. The second experiment resembled more closely the conditions under which the drug is applied in human medicine. This type of experiment, however, introduces additional variable factors such as the amount of hæmolysin liberated in animals receiving the same dose of streptococcal culture; this renders the interpretation of results much more difficult than in the first experiment, in which an exact amount of antigen was injected. It was probable that some such variation in streptolysin production in the two foci in rabbit 14 accounted for the suggestion of an inhibitory action of prontosil. This was the only example of the kind encountered in the investigation.

The observations may throw further light on the disappointing results with prontosil in the treatment of acute rheumatism reported by Swift *et al.* (1938). If, as suggested by Coburn (1936), the delayed and precipitous rise in A.S.O. titre after hæmolytic streptococcal infection of the throat is part of the rheumatic process, then prontosil therapy will neither prevent nor reduce the antibody response. On the other hand, the prophylactic use of prontosil in

preventing throat infection and thereby eliminating the most common stimulus for increase in ASO titre should be of benefit. Encouraging results for this method of prophylaxis have already been reported by Coburn and Moore (1939).

A much more remote question upon which the work touches is that of immunisation against hæmolytic streptococcal infection. If it is possible so to develop control of hæmolytic streptococcal infection by means of prontosil that all risk of spread of infection is eliminated or reduced to low limits it may also be possible to introduce a system of modified infection with virulent organisms for improving passive and possibly active immunisation against certain of the diseases of which this organism is the causative agent.

SUMMARY

1 The effect of prontosil on the production of antistreptolysin O in rabbits has been investigated.

2 No evidence of inhibition or stimulation of antibody response was obtained.

3 The serum of non immune rabbits under prontosil treatment had no neutralising action on streptolysin O, nor were the anti-hæmolytic O titres of immune rabbits increased by the treatment.

4 The results are discussed in relation to the use of prontosil in rheumatic fever.

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OBSERVATIONS ON THE ANTISTREPTOLYSIN O TITRE IN RELATION TO THE MECHANISM OF ACUTE RHEUMATIC FEVER

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The antigenic nature of the hemolytic filtrates of streptococcal cultures in serum free broth was described by Todd (1932a) who later (1934) demonstrated the group specificity of the products and detailed the titration of a neutralising antibody to be distinguished in further work as anti streptolysin O. Todd (1938 a and b, 1939) found that group A (Lancefield 1933) hemolytic streptococci produce two serologically distinct varieties of hemolysin one designated streptolysin O on account of oxygen sensitivity and the other streptolysin S in virtue of extractability in serum. The response in animals to experimental infection with whole cultures of group A hemolytic streptococci was a rise in titre of the neutralising antibodies, anti-streptolysin O (A S O) and anti-streptolysin S. Todd's later work has not been published. O titration in the

Todd (1932b) first reported the A S O titres of normal human sera to be in the zone of 3-100 units and demonstrated a rise in titre of varying degree after known streptococcal infections such as scarlatina, erysipelas, etc. Coburn and Pauli (1935a) supported these observations by reporting that student nurses before exposure to hemolytic streptococcal infection had A S O titres of from 33 to 100 units, the mean for the group being 83. In nurses who contracted infection, the titres were later found to range from 117 to 333 units the mean being 200, while in nurses who escaped infection they remained at the original low level. In a later communication the same authors (1935b) noted that the mean of the A S O titres in 176 individuals in good health was 71 units.

On the basis of these and similar results, the titration of A S O has been applied to the study of streptococcal infection in acute rheumatism. Todd (1932b) reported a rise in titre during the active stage of rheumatic fever but considered that this was dependent on the frequent antecedent infection of the throat by hemolytic streptococci and not necessarily on the intensity of the rheumatic process. Coburn and Pauli (1932) observed a precipitous rise in titre which immediately preceded the appearance of rheumatic manifestations and concluded that the rheumatic attack had been initiated by hemolytic streptococcal infection. Studying the results of a wave of infection in a group of rheumatic children all in the quiescent phase with carditis, Coburn and Pauli (1935 c and d) recorded that 14 out of 16 individuals infected with the epidemic strain developed acute rheumatism, the onset of symptoms being accompanied by a coincident rise in A S O titre. Seven children who escaped infection also failed to develop acute symptoms and



Type of acute rheumatism

The cases of acute rheumatism under investigation were mainly primary attacks in boys who had passed a certain standard of medical examination not longer than three years prior to the onset of illness. With few exceptions, anyone admitting a previous history of rheumatism had had no other recurrence within that period.

Acute rheumatism manifested itself in the group with the usual symptoms of pain and stiffness in one or more joints, followed by flitting arthritis in a varying number of joints. Marked effusions were not common but transient swelling and tenderness were present in the early stages. Cardiac complications followed in a considerable proportion of cases. Although the joint symptoms were generally of moderate severity, the cardiac lesions rendered the condition no less serious.

Clinically the cases were arbitrarily divided into monocyclic and polycyclic types. The monocyclic group presented a single phase of activity with pyrexia and joint pains of varying degree and duration, with or without cardiac complications. In polycyclic cases, two or more such attacks were separated by less interse periods in which there was some indication of continuing activity.

Range of antistreptolysin O titre in acute rheumatism

The range of antistreptolysin O titre during the active and inactive phases of 110 acute rheumatic attacks is presented in table I. The cases were divided according as the clinical picture was of the monocyclic or polycyclic form. The results were next grouped according to the extent of the titre range, i.e. the difference between the highest and lowest readings recorded throughout the particular attack. In the modified test, the new arrangement was such that the increment from one dilution to the next is the same irrespective of the titre level. In other words, the logarithms of the units increase in an arithmetic series. The group showing *maximum variation* included all cases with a titre range represented by a difference in end point of 6 or more tubes in the neutralisation test or titration. The *moderate variation* group was similarly represented by a difference in end point of 3.5 tubes. Variations in titre in these two groups could be considered significant. The *minimum variation* group included all cases whose serial titrations remained at a constant level. Also included in this group were cases in which differences of only 1.2 tubes existed, these being considered insignificant under the conditions of the test.

Table I shows that 56 of the 64 monocyclic cases were accompanied by a significant change in titre. This figure included 50 cases in which titres during the active phase of rheumatism were higher than in the inactive phase, and 4 in which the titres

during inactivity were the higher. No titre below 100 units was recorded in the monocyclic cases with constant titres, 5 being in the 100-249 zone and 3 at 250 units or over.

TABLE I

The range of antistreptolysin O titre (units per c.c.) in scarlatina, hæmolytic streptococcal pharyngitis and the monocyclic and polycyclic forms of acute rheumatism

Range of antistreptolysin O titre	Acute rheumatic cases						Non-rheumatic cases	
	Clinical type						Scarlatina	Pharyngitis
	Monocyclic		Polycyclic		All cases			
	No.	Per cent.	No.	Per cent.	No.	Per cent.		
Maximum variation								
Titres higher in active phase .	22	34.5	27	58.7	49	44.5	11	7
Titres lower in active phase .	0	0	0	0	0	0	0	0
Titres varying in same phase .	1	1.5	4	8.7	5	4.5	0	0
	23	36.0	31	67.4	54	49.0	11	7
Moderate variation								
Titres higher in active phase .	28	43.7	11	23.9	39	35.4	4	8
Titres lower in active phase .	4	6.3	0	0	4	3.6	0	0
Titres varying in same phase .	1	1.5	1	2.2	2	1.8	0	0
	33	51.5	12	26.1	45	40.8	4	8
Minimum variation								
Titre range in zone of 0.99 .	0	0	2	4.3	2	1.8	0	0
Titre range in zone of 100-249	5	7.8	1	2.2	6	5.5	0	7
Titre range in zone of 250 and over .	3	4.7	0	0	3	2.8	0	2
	8	12.5	3	6.5	11	10.1	0	9
	64	100.0	46	100.0	110	99.9	15	24

In the polycyclic series, 43 of 46 cases showed a significant variation. In 38 the active phase titres were higher than the inactive, and in no case were all the inactive titres higher, although significant change in titre occurred without detectable clinical alteration in 5 cases. Of the 3 cases with fixed titres in all stages, 2 were in the 0.99 unit zone and 1 in the 100-249.

Thus 88 of 110 cases showed a response indicative of recent streptococcal infection, 11 failed to react and 4 cases presented a reversed effect.

Range of antistreptolysin O titre in scarlatina and in hæmolytic streptococcal pharyngitis

From each of 15 cases of scarlatina and 24 cases of hæmolytic streptococcal pharyngitis, a sufficient number of titrations were available to determine the range during active infection and

convalescence (table I). In scarlatina, all titres during activity were higher than in convalescence, 11 of the 15 cases exhibiting a maximum variation. Of the 24 cases of pharyngitis, a maximum reaction was noted in only 7, and in 8 cases a moderate increase occurred during activity. The remaining 9 cases showed no appreciable change although all the titres were over 100. As sera from several of these cases were not obtained until 4-7 days after the onset of infection, it is possible that an early reaction was missed.

For purposes of comparison the data were next examined in the manner followed by Todd *et al.*

Distribution of antistreptolysin O titres in various clinical groups

The combined titration results of sera from various clinical sources are presented in table II.

Healthy subjects. As previously indicated the sera from 107 healthy subjects were selected at random without regard to history of recent pharyngitis for which there were no bacteriological data. The distribution of the A.S.O. titres in this group (table II, col. 2) was such that 88 were less than 125 units and only 3 above 250, the arithmetic mean being 79.

Non-rheumatic pharyngitis and scarlatina. The combined distribution of these two groups (148 sera, col. 5) represented a marked shift to higher figures, 107 being over 125 units and 68 above 250, the arithmetic mean being 280. The scarlatina figures (col. 4) were somewhat higher than those for simple pharyngitis (col. 3), the arithmetic means being 300 and 263 respectively.

Rheumatic group. The general effect of rheumatic activity was obtained by comparing the distribution of all sera collected during active and inactive periods (cols. 8 and 9). The distribution of titres during quiescent periods was intermediate between that of the normal controls and the non-rheumatic pharyngitis and scarlatina group but much nearer the latter. The A.S.O. titres of 263 of 398 sera (66 per cent.) taken during the inactive period were 125 units or more, and 138 were over 250 units. With the development of rheumatic activity, the distribution was raised much higher, 629 of 693 sera (90.7 per cent.) being over 125 units and 484 sera (69.7 per cent.) above 250. Whereas in only 13 sera (3.2 per cent.) during inactivity was the titre 625 units or more, the active group produced 158 (22.7 per cent.) at this high level. The arithmetic means of the active and inactive groups were 444 and 210 units respectively.

Pharyngitis in rheumatic subjects. While under observation, 20 rheumatic subjects in the quiescent state contracted hæmolytic streptococcal pharyngitis. Following upon the throat infection, 16 of the 20 patients developed acute manifestations. Seventeen

attacks were followed, one subject having two such lapses, and the results are collected in table II (col. 7). The distribution of the titres of 61 sera from this group was very similar to that in the active rheumatic series (col. 8), as were the arithmetic means.

TABLE II
*Distribution of antistreptolysin O values observed in sera
of different groups*

Antistreptolysin O units per c.c. of serum Col. 1	Non-rheumatic				Rheumatic			
	Healthy controls Col. 2	Pharyngitis Col. 3	Scarlatina Col. 4	Pharyngitis and scarlatina Col. 5	Pharyngitis		Active periods Col. 8	Inactive periods* Col. 9
					No rheumatic attack Col. 6	With rheumatic attack Col. 7		
Absolute numbers of sera observed								
16-82	70	19	7	26	3	7	41	73
83-124	18	11	4	15	4	2	23	62
125-249	16	19	20	39	7	9	145	125
250-399	2	14	18	32	4	18	205	85
400-624	1	12	5	17	0	10	121	40
625-832	0	3	5	8	0	11	61	7
833-999	0	0	0	0	0	0	18	1
1000 or more	0	5	6	11	0	4	79	5
Total sera	107	83	65	148	18	61	693	398
Average unit- age per c.c. serum	79	263	300	280	188	420	444	210
Percentage frequencies								
16-82	65.3	22.7	10.8	17.5	...	11.5	5.9	18.3
83-124	17.0	13.5	6.2	10.0	...	3.2	3.3	15.6
125-249	14.9	22.9	30.8	26.4	...	14.7	21.0	31.4
250-399	1.9	16.8	27.8	21.7	...	29.5	29.6	21.4
400-624	0.9	14.6	7.6	11.5	...	16.4	17.4	10.0
625-832	0	3.5	7.6	5.4	...	18.0	8.8	1.7
833-999	0	0	0	0	...	0	2.6	0.3
1000 or more	0	6.0	9.3	7.4	...	6.6	11.3	1.2
Percentage frequencies in condensed form								
16-249	97.2	59.1	47.8	53.9	...	29.4	30.2	65.3
250-624	2.8	31.4	35.4	33.2	...	45.9	47.0	31.4
625 or more	0	9.5	16.9	12.8	...	24.6	22.7	3.2

* Pre- and post-activity.

Four patients experienced streptococcal pharyngitis without re-awakening rheumatic infection, and in all four there was no rise

in A S O titre Only 18 sera were available from this group which were too few for purposes of significant comparison It may be noted, however, that in 14 of the 18 sera the titres were less than 250 units and none was above 400 (col 6)

Degree of rheumatic activity

In table III the results are grouped according to the degree of clinical activity Only 12 sera were obtained during the period immediately preceding activity, all being below 400 units, and the

TABLE III

The distribution of antistreptolysin O values observed in sera during a rheumatic attack, according to the degree of rheumatic activity

Antistreptolysin O units per c.c. of serum	Rheumatic activity				
	Pre active	Increasing	Maximum	Decreasing	Doubtful
Absolute numbers of sera observed					
16-82	4	0	12	11	11
83-124	1	1	1	13	7
125-249	1	9	31	35	50
250-399	6	12	43	92	16
400-624	0	5	37	73	26
625-832	0	4	19	25	13
833-999	0	2	7	8	1
1000 or more	0	1	11	33	14
Total sera	12	34	181	290	181
Average titre (units)	167	412	538	450	363
Percentage frequencies					
16-82	33.4	0	6.6	3.8	7.7
83-124	8.3	2.9	0.6	4.5	3.9
125-249	8.3	26.4	17.1	19.0	27.6
250-399	50.0	35.3	23.8	31.7	31.0
400-624	0	15.7	20.3	18.2	14.4
625-832	0	11.8	10.5	8.6	7.2
833-999	0	5.9	3.9	2.7	0.5
1000 or more	0	2.9	17.1	11.4	7.7
Percentage frequencies in condensed form					
16-249	50.0	29.3	24.3	27.3	39.2
250-624	50.0	50.0	44.1	49.9	45.4
625 or more	0	20.6	31.5	22.7	15.4

mean, 167, was the lowest in any phase of the illness During increasing activity the distribution shifted up the scale, the mean titre reaching 412 units The peak was attained at the stage of

greatest activity with a mean of 538 units. Even at this stage, 12 specimens were below 82 units. With diminishing activity, the figures tended to return to lower values but remained at relatively high levels as compared with those in the quiescent phase (*cf.* table II). The arithmetic mean in the immediate post-active phase was 363.

*Effect of length of time since infection in scarlatina
and pharyngitis*

From cases of scarlatina only 6 sera were taken in the first 6 days of the attack. The titres of 5 were less than 125 units and the remaining titre was 159 (table IV). In the second week of the

TABLE IV

Distribution of antistreptolysin O values observed in sera from scarlatina and haemolytic streptococcal pharyngitis, according to duration of time since infection

Antistreptolysin O units per c.c. serum	Scarlatina				Pharyngitis				Scarlatina and pharyngitis			
	Interval in days from start of illness											
	0-6	7-13	14-21	22 or more	0-6	7-13	14-21	22 or more	0-6	7-13	14-21	22 or more
Absolute numbers observed												
0-82	4	2	0	1	9	4	2	4	13	6	2	5
83-124	1	0	0	3	4	3	0	4	5	3	0	7
125-249	1	3	9	7	6	2	6	5	7	5	15	12
250-399	0	1	6	11	1	1	7	5	1	2	13	16
400-624	0	0	4	1	2	3	3	4	2	3	7	5
625-832	0	0	3	2	1	0	1	1	1	0	4	3
833-999	0	1	0	0	0	0	0	0	0	1	0	0
1000 or more	0	0	4	1	0	1	3	1	0	1	7	2
Condensed table												
0-124	5	2	0	4	13	7	2	8	18	9	2	12
125-249	1	3	9	7	6	2	6	5	7	5	15	12
250-624	0	1	10	12	3	4	10	9	3	5	20	21
625 or more	0	1	7	3	1	1	4	2	1	2	11	5
Percentage frequencies												
0-124	83.3	28.6	0	15.4	56.5	50.0	9.1	33.3	62.0	42.9	4.2	24.0
125-249	16.6	42.9	34.7	26.9	26.1	14.3	27.3	20.8	24.2	23.8	31.2	24.0
250-624	0	14.3	38.4	46.1	13.1	28.6	45.4	37.5	10.3	23.8	41.8	42.0
625 or more	0	14.3	26.9	11.6	4.3	7.0	18.2	8.3	3.4	9.5	22.9	10.0

illness, 5 of 7 specimens had a titre of more than 125 units, and in the third week the highest distribution was observed, 17 of 26 (65.3 per cent.) having a titre of 250 or more. The figures during

the fourth and subsequent weeks showed a commencing regression to lower values

The distribution in simple pharyngitis approximated closely to that in scarlatina with only minor differences. Thus higher titres were observed at the commencement of the illness, but in the following weeks the distribution was at a slightly lower level than in scarlatina. Nevertheless, the highest distribution was again observed during the 3rd week. This can be correlated with the finding that 9 of the pharyngitis cases (table I) started with titres over 100 but showed no increase, whereas all the scarlatina cases responded with a marked increase in titre.

Titration curves in monocyclic cases of acute rheumatism

Examples of the titration curves are illustrated in fig 1. Cases 2, 3, 4, 6 and 7 correspond to those described by Coburn (1936a) as typical of the rheumatic state in that the maximum titre was reached after the period of greatest activity was passed and when the sedimentation rate was improving. Abnormally high titres were maintained for weeks, and in some instances for several months, but a final decrease was observed. In other cases, as in 1 and 5 (fig 1), the titres were as high at the onset of the attack as in any subsequent stage. It could not be stated that only severe cases were accompanied by a prolonged ASO response. Thus case 3 was mild and of short duration, without detectable cardiac complications, but the titre remained at a very high level for several months. On the other hand all severe cases in which any rise in titre developed, presented this to a high degree. As in all other cases in this group, a final fall in titre was observed.

Cases 8-11 constituted the small group (4 out of 64) in which titres during inactivity were higher than in the active phase. In case 8, no significant shift in the titre from a resting level of 100 accompanied a severe attack, but in the 12th week, when convalescence was well established, the titre rose to 250 and was maintained at that level until observation ceased in the 20th week. Case 9 started with a titre of 50 at the onset of an attack of short duration. At the end of the 4th week, an increase to 625 was noted and was succeeded by a drop to 200 in early convalescence. A progressive increase followed which finally surpassed and remained above the highest titre during activity. There was no clinical reason for this increase in titre, the patient remaining perfectly fit. Cases 10 and 11 were similar to 9, save that no fall in early convalescence was noted. As a significant increase occurred during the attack in cases 9-11, they possibly represented extreme examples of delayed reaction, but no correlation of antibody response with activity of the rheumatic process could be made. The titres of cases 8-11 remained close to the final levels recorded in fig 1 until

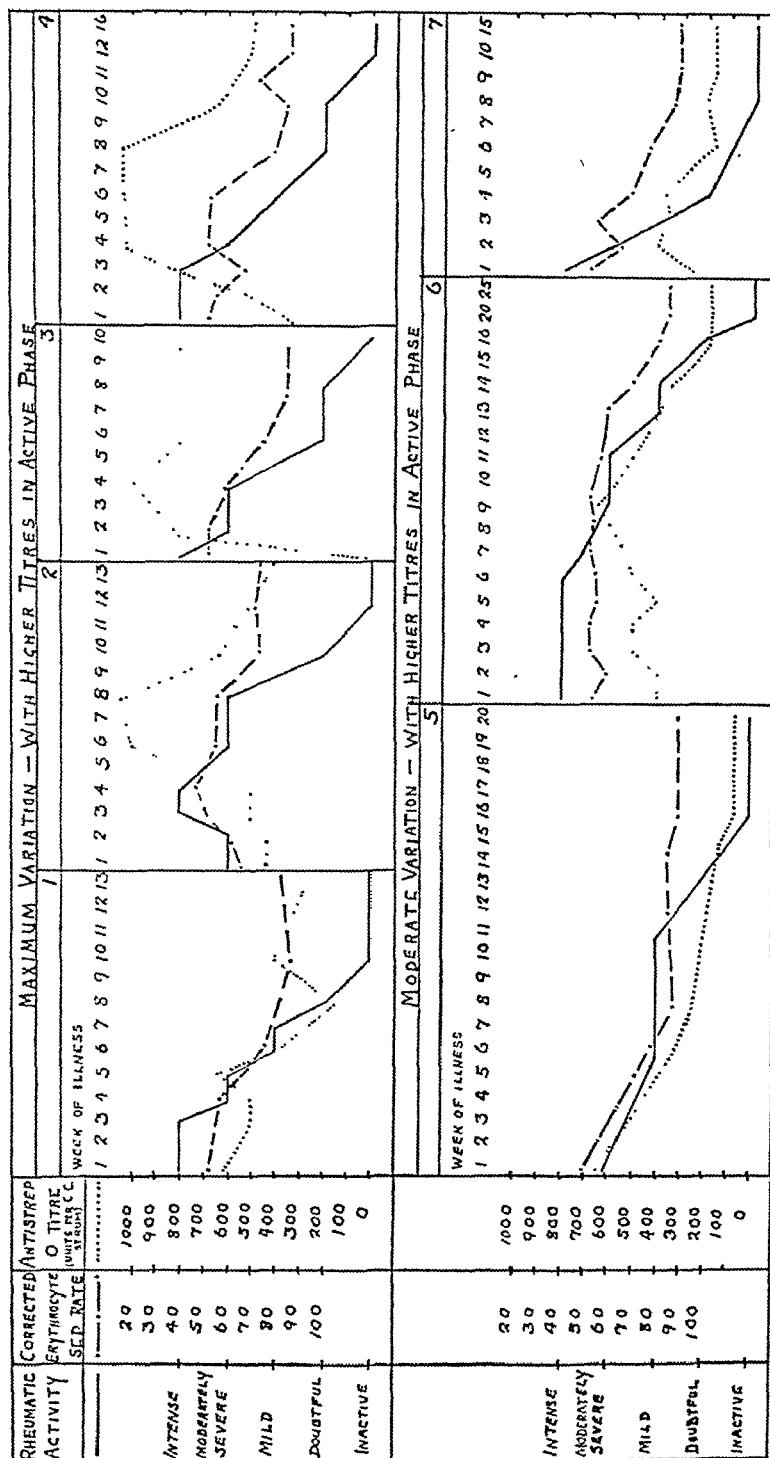


FIG. 1.—To show examples of variation in antistreptolysin O response in monocyclic attacks of acute rheumatic fever.

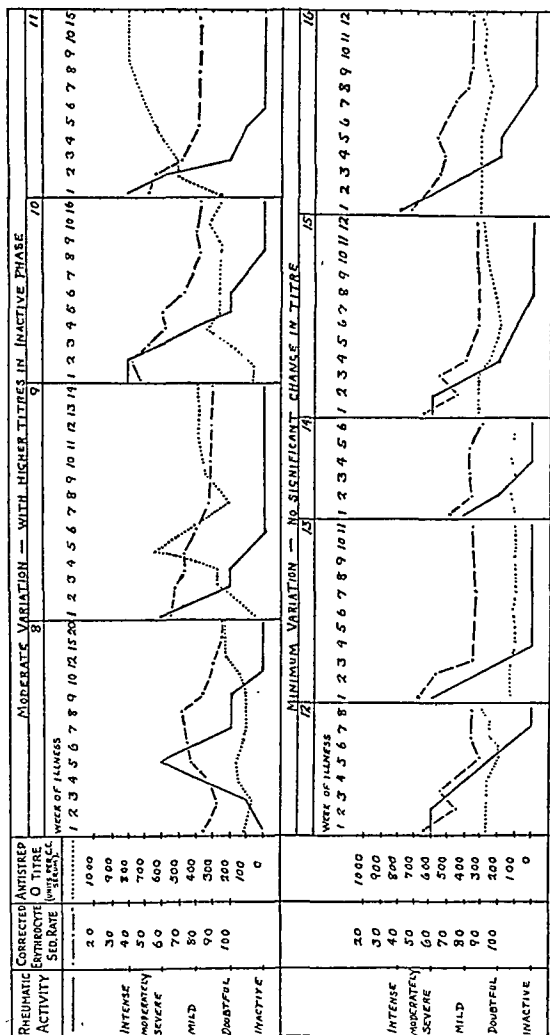


FIG. 1.—Continued.

the end of an observation period of 6 months. It was therefore considered advisable to place them in a separate serological category to differentiate them from the majority of cases in which a final fall was noted before the end of a similar period, although no clinical distinction between the two groups could be made.

Cases 12-16 were representative of the group (8 out of 64) in which no significant change in titre was detected throughout the attack. Clinically, these cases were also indistinguishable from the majority in which an increase had occurred. Case 16, for example, was a severe polyarthritis affecting many joints before the condition finally settled down. The titre was 312 at the first observation on the 2nd day of illness and remained at that level throughout. As in cases 12 and 14, the titres were well above the mean of 79 observed in the normal control group. The other cases illustrated were of a less severe nature, but nevertheless bore the typical character of rheumatic polyarthritis.

Titration curves of polycyclic cases of acute rheumatism

As shown in fig. 2, these cases afforded good examples of the relationship between rise in titre and clinical crises. In cases 17 and 18, three clinical cycles were each attended by an advance in titre, although this was not always of the same intensity. Thus in case 17 the first two cycles were followed by marked responses but the third produced a much smaller reaction. In other cases, as in nos. 20-22, the swing in titre between the cycles was not so evident and the titre curve remained at a high level throughout the active phase of illness.

The small group of 3 cases in which there was no significant change in titre was formed by cases 24-26. Of these the first two were clinically severe and 26, though of milder character, persisted for 21 weeks with titres varying no more than in the period charted.

Variation in titre without alteration in clinical state

On account of their clinical reaction, those cases showing marked variation in titre during the same clinical phase are separately illustrated in fig. 3. The two monocyclic cases, 27 and 28, both had titres in early convalescence lower than those obtaining in the active stage, followed by a rise in late convalescence. The polycyclic series supplied 4 cases in this category which were observed for longer periods. The response of each was similar in that the first cycle of the attack was accompanied by a sharp rise in titre followed by a rapid fall. One case, no. 30, showed an insignificant response to subsequent cycles but the remaining 3 cases produced no such rise, although the clinical cycles were as intense as that at the onset of illness.

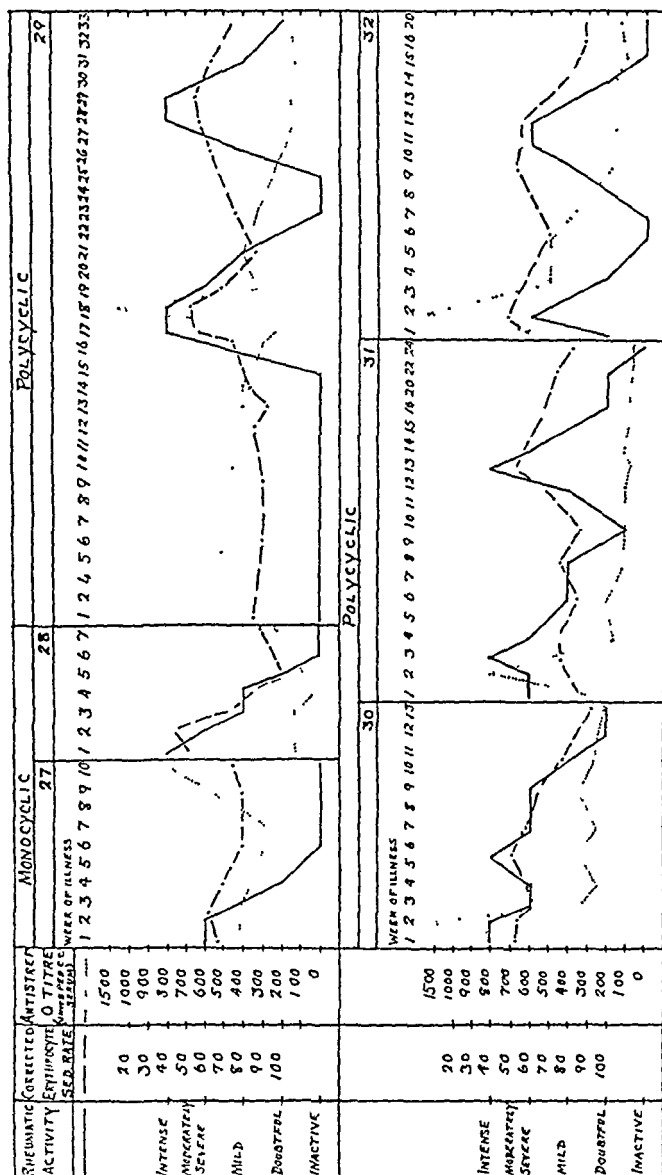


FIG. 3.—To show examples of variation in antistreptolysin O titre during active and quiescent phases of monocyclic and polycyclic attacks of acute rheumatic fever.

Antistreptolysin O response in scarlatina and in hemolytic streptococcal pharyngitis

Statistical examination had shown that increase in titre was invariably noted in the cases of non-rheumatic scarlatina studied, the mean being raised to 300, with maximum titres occurring in

the 3rd and 4th weeks of infection. In simple pharyngitis due to *Streptococcus hemolyticus*, there was usually but not always a similar response, the mean being 263.

Selected cases are illustrated in fig. 4. Of the scarlatina group, cases 1 and 2 were typical examples of the intense but rapid reaction during the 2nd and 3rd weeks. Cases 3 and 4 exemplified the occurrence of maximum titres in the 4th and 5th weeks. In pharyngitis there was also considerable variation in response from the high, rapidly attained peak in case 1 to the slow weak response in case 4. The absence of any change in titre noted in 9 of the 24 pharyngitis cases examined is illustrated in case 5.

There was considerable individual variation, therefore, in this non-rheumatic group after streptococcal infection. No prolongation of the increased ASO titre was seen, although the actual appearance of increase was not apparent in some cases until the 4th or 5th week. The absence of reaction in a minority of cases indicated that a negative result could not be considered certain evidence of the non-occurrence of recent streptococcal infection.

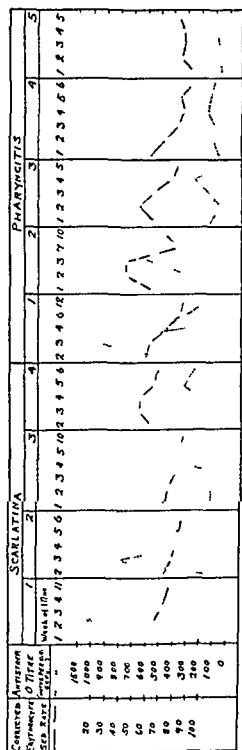


FIG. 4.—To show examples of antistreptolysin O curves in scarlatina and in hemolytic streptococcal pharyngitis (non rheumatic).

Hemolytic streptococcal pharyngitis in rheumatic subjects

While under observation, 20 rheumatic subjects in the quiescent phase contracted pharyngitis. Four attacks were followed by uninterrupted convalescence and in all four cases there was no increase in ASO titre. Fourteen of the 18 sera from this group had titres of less than 250. One case was observed through two attacks of pharyngitis, of which only the second was followed by mild rheumatic activity. The first throat infection which failed to

stimulate rheumatic infection also failed to produce any increase in titre, but the second attack of pharyngitis was accompanied by a moderate rise which persisted during the subsequent period of clinical activity.

Further exceptions were sought by noting instances of renewed activity in rheumatic subjects who had no antecedent pharyngitis.

Reactivation of rheumatism without pharyngitis

While under observation, 10 subjects in whom rheumatic activity had been quiescent for varying periods developed acute manifestations without clinical evidence of pharyngitis. There were no subjective complaints and no abnormality of the throat was detected in routine examination. In 7 of these cases, hæmolytic streptococci were isolated from the throat before the onset of symptoms, either for the first time or after a series of negative results, and in 6 of these the attack was followed by a significant rise in A.S.O. titre. In the remaining 3 cases, hæmolytic streptococci were not isolated from the throat in repeated examinations but two of these cases also showed a significant rise in titre. In this group of 10 cases, rheumatic activity was accompanied by a rise of titre in 8, despite the absence of clinical evidence of pharyngitis before or at the onset of the attack.

DISCUSSION

The results from the clinical groups were particularly suitable for purposes of comparison in that all the subjects were males in a strictly limited age-group of 16-18 years.

The healthy controls were males in a slightly higher age-group of 18-20 years. The low A.S.O. values of sera from this group closely followed the results of Coburn and Pauli (1935a), the respective means being 79 and 83. Although controls in the 16-18 years age-groups were not available, little difference was to be expected from the distribution found in the older boys who had been in a similar environment for at least two months before examination. If anything, the readings in younger boys may have been lower on account of reduced opportunities in age-years for infection to have occurred. On the other hand, it may be argued that there did exist environmental differences in the exposure of the clinical groups and healthy controls, resulting in a greater risk of infection so that the initial titres of the former were higher. Apart from the absence of any evidence of such environmental differences, this criticism is countered by the low titres of the scarlatinal and pharyngitis groups in the first week of illness and by the relatively low titres of the small number of sera from rheumatic patients prior to the onset of activity.

The distribution of titres in non rheumatic scarlatina and pharyngitis presented, therefore, a marked and significant shift up the scale of values but to a lower plane than that observed by Todd *et al* (1939). The arithmetic mean was actually higher, namely 280 as against 239, but this was due to the presence of fewer extreme values. The distribution approximated more closely to that of the rheumatic group with pharyngitis but no rheumatic activity in Todd's series.

The data indicated that the maximum titre in scarlatina and pharyngitis was attained most frequently in the period 14-21 days after infection. Experimentally it has been observed that the A S O titre falls within a few days of supplying the antigen (Green, 1941). From these observations it may be deduced that even in simple pharyngitis and in scarlatina there is prolongation of the antigenic stimulus for a considerable time after the acute throat infection has subsided, but that this does not extend beyond 4-5 weeks. Coburn and Pauli (1939) have fully demonstrated that prolongation of the increased titre in scarlatina and pharyngitis was due to complications or continued infection.

The results confirm previous observations on the general increase in A S O titre in acute rheumatism, although the degree of increase was not as great as that reported by Todd *et al*. In presenting the results, two methods were considered essential in attempting correlation with the clinical process. The tabular recording of actual titre values provided a very satisfactory method for the statistical comparison of different groups but did not disclose the various responses encountered in a single clinical entity. Thus although, in a recognised infection such as hæmolytic streptococcal pharyngitis, an increase in A S O titre was the general finding, some patients failed to respond, irrespective of the high or low level of the initial titre. No fall in titre, however, was noted in an established infection. As may be expected from the toxigenic nature of scarlatina strains of hæmolytic streptococci, six fully observed cases of this disease presented a well marked reaction.

The variations in non rheumatic subjects must be considered when the A S O response of rheumatic subjects is examined. The shift to higher values in rheumatism was confirmed, as was the maintenance of high titres for much longer periods than in the non rheumatic controls. Therefore it was surprising to find that 6 out of 17 acute attacks in known rheumatic subjects, preceded by pharyngitis of hæmolytic streptococcal origin were not accompanied by any significant increase in titre. This proportion, it may be noted, was roughly that of the non rheumatic group, whose titres were also unaffected by pharyngitis. Reactivation of rheumatism, therefore, could not be invariably linked with further increase in A S O titre following repeated throat infection.

However, it should be remembered that the distribution of A.S.O. titres in inactive rheumatism was much higher than in normal controls and therefore the above conclusion did not exclude the possibility of further streptococcal activity which was masked by the high titres already reached. Unpublished observations have shown that in animals with relatively high A.S.O. titres the further injection of large amounts of streptolysin O may have little or no effect. When the variation in non-rheumatic subjects to hæmolytic streptococcal infections was taken into account, the evidence in support of such infection in the majority of the rheumatic group was considerably strengthened. In the absence of direct proof of continued streptococcal infection, Coburn (1936a) has suggested that this evidence indicated an altered response on the part of the rheumatic subject to antecedent infection. One difficulty in the acceptance of this theory of mechanism has been stressed by noting that typical attacks recur in known rheumatic subjects without further increase in titre.

Although direct proof of continued infection has not been obtained, Green (1939) suggested this possibility in reporting that hæmolytic streptococci had been recovered from heart-valve cultures made *post mortem* in cases of acute rheumatism. The source of the organisms in such material could not be definitely ascertained but the strain in each case was of the same serological type as that isolated in life from the throat, which appeared to exclude an extraneous origin. Heart-blood cultures were all negative for hæmolytic streptococci, although terminal infection of the blood could not be absolutely excluded as a possible explanation for the presence of the organisms in heart-valve cultures. Thomson and Innes (1940) have since confirmed this observation and Collis (1939) has also reported similar results.

While the importance of hæmolytic streptococcal infection has been emphasised, there still remains for consideration the minority of cases of acute rheumatism in which no increase in A.S.O. titre was observed. Either these were similar in nature to non-rheumatic cases which failed to react to infection by demonstrable antibody production, or else such infection had no part in inducing their rheumatic state. With the evidence at present available it is impossible to exclude either alternative, but concentration of investigation on cases in this group should yield important information in regard to ætiology.

CONCLUSIONS

1. The antistreptolysin O titres of 1346 sera from male adolescents, including various clinical groups and healthy subjects, have been determined.

2 The mean titre in normal controls was 79, in 82.3 per cent of the sera the titre was less than 125 units

3 An increase in titre was invariably noted in scarlatina, the mean being raised to 300, with maximum titres occurring in the 3rd and 4th weeks of infection

4 Simple pharyngitis due to *Streptococcus hæmolyticus* was usually but not always followed by a rise in titre, the mean being 263

5 The mean titre in the active phase of acute rheumatism was 444 and during the inactive phase 210

6 Of 110 attacks of acute rheumatism, 79.9 per cent were accompanied by a significant increase in titre which, in the majority of cases, reached maximum proportions at or just after the height of clinical activity, in 10.1 per cent of attacks no change in titre was observed and in 3.6 per cent the titres were reduced during the active phase

7 Examples of the various types of antistreptolysin O response in rheumatic and non rheumatic subjects are described

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REVERSED PASSIVE SKIN SENSITISATION TO HORSE SERUM IN HUMAN BEINGS

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THE development of anaphylactic shock in animals as the result of the parenteral injection first of an antigen and later of a serum, either homologous or heterologous, containing the appropriate antibody, has been demonstrated by Opie (1924), Combesco and Branner (1928), Kellott (1935), Zinsser and Ender (1936) and van den Ende (1940). This form of shock, which is not so consistently reproducible as that in the better recognised condition of passive anaphylaxis, is now usually known as reversed passive anaphylactic shock. Both forms are, however, believed to be fundamentally similar in that they result from the damaging effects of the combination of antigen and antibody either in the interior or on the surface of certain tissue cells. Though the number and variety of the antigens so far used in attempts to elicit reversed passive anaphylactic reactions have been limited, certain failures have been observed, and it has been suggested (van den Ende) that in these instances the antigenic protein is 'unacceptable' to the animal injected. When

to the animal used, it is the antigen or the antibody first comes into contact with the reactive tissue cells and sensitises them for the later advent of the other.

The local as opposed to the general transference of passive sensitisation in human beings was clearly demonstrated by Prausnitz and Kustner (1921) when they successfully sensitised the skin of a normal subject by injecting intradermally a small quantity of serum from a man highly sensitive to fish muscle proteins. This type of reaction, which has since been extensively studied, corresponds on a diminutive scale to the ordinary form of passive anaphylactic shock, though it is not yet universally agreed that the two reactions have the same fundamental underlying mechanism. The converse reversed passive sensitisation of the skin has been effected by Opie, though to do so he injected subcutaneously or intravenously what would now be regarded as very large quantities of horse serum to sensitise rabbits to a subcutaneous injection of rabbit or horse antiserum twenty-four hours later. The interaction of antigen and antibody in the subcutaneous tissues led to the development of severe local inflammatory oedema, which was marked on the following day and in some instances persisted for several days. Opie does not mention how soon after the second injection the reaction set in.

In the present series of experiments the reversed passive sensitisation reaction of the skin of human beings has been examined by injecting intradermally a very small quantity of horse serum—which contains at least one and possibly more acceptable antigenic

proteins—and after an interval injecting into the same site a small quantity of serum from a human subject who was very sensitive to horse serum. By analogy, this type of reaction would correspond with the reversed passive anaphylactic shock described by Opie and others, and its underlying mechanism may possibly bear the same relation to this form of anaphylaxis as the Prausnitz-Küstner reaction bears to the ordinary passive anaphylaxis. Whatever may be their mechanism, both these local skin reactions resemble one another so closely as to be indistinguishable in appearance and clinical course.

METHODS AND SOURCES OF MATERIAL.

The horse serum used was tetanus antitoxin (natural serum) prepared by the Lister Institute and containing 0.35 per cent. of tricresol. In the dilutions used, intradermal injection of this serum caused only slight reactions in the majority of human subjects.

The human antisera were obtained from two subjects who were highly sensitive to horse serum. Both had been treated on several occasions for prophylactic or therapeutic reasons with antitoxin-containing horse serum, and on the last occasion both had developed severe general reactions. One serum (J.E.S.), which was used in all the experiments except those summarised in table II, when undiluted gave ring-test precipitin reactions with horse serum in concentrations from 1:5 to 1:20; the other (G.M.C.) failed to give any reaction from 1:5 to 1:320.

Normal human sera were obtained from two men both of whom, after their blood had been taken, failed to react to an intradermal injection of 0.1 c.c. of a 1:10 dilution of horse serum. No preservatives were added to any of the human sera used and no specimen was more than two weeks old at the time of injection.

Nearly all the subjects passively sensitised were medical students aged 20-26 years: three were over 30 years. No subject was used for more than one experiment and none of the few that reacted to the preparatory injection of horse serum was later injected with human serum. The injections were made into the flexor surface of the upper half of the forearm. Both human and horse sera were injected as nearly as possible at the same point in the skin and in the same direction, so that both solutions should infiltrate the same area.

The reactions were recorded by tracing on celluloid film the outline of the area of confluent erythema that developed in 15-20 minutes, when the flare reaction was maximal, and transferring the outline to squared paper from which the area could be determined. In some of the experiments the size of the wheal also was recorded in this way.

RESULTS

In most of its clinical features the present reaction closely resembles the Prausnitz-Küstner reaction. The intradermal injection of the antiserum into skin sites sensitised 24-48 hours previously with horse serum was followed 3-5 minutes later by a growing area of erythema round the point of injection. As with the Prausnitz-Küstner reaction this was at first punctate, but as the flare developed most of the punctate areas grew until the innermost became

confluent Even at the height of the reaction, however, usually in 15-20 minutes, there were always a number of small discrete areas of erythema several mm outside the central confluent flare The flare extended more or less evenly in all directions, finally forming a rough circle or broad oval with the point of injection near the centre

In positively reacting areas the small rounded elevation in the skin which resulted from the intradermal injection gradually enlarged, became flattened on the top and assumed the characteristic appearance of a skin wheal When normal human serum and antiserum were injected separately into symmetrical prepared sites on the two arms, the small skin elevations due to the injections were at first indistinguishable, but after 10-15 minutes they contrasted strongly that raised by the normal serum had largely subsided, while that produced by the antiserum had enlarged to several times its initial size and often showed pseudopodial spreading

The entire reaction, including both erythema and wheal, faded almost completely in about 2 hours, the erythema disappeared first and the wheal gradually became softer and more diffused No recrudescence was observed

Comparison of the size of the reactions caused by normal serum and antiserum

Eleven subjects were prepared by four preliminary intradermal injections of 0.1 c.c. of diluted horse serum into two points about 5 cm. apart in each upper forearm At the upper point the concentration used was 1:20, at the lower, 1:100 After 48 hours 0.1 c.c. of antiserum was injected intradermally into the two prepared sites on the left arm and also into a third previously untreated site about 5 cm. lower and rather more medial in the same arm The two prepared sites and a third unprepared site in the right arm were similarly injected with 0.1 c.c. of normal human serum The third injection in each arm was for the purpose of excluding the possibility that the human sera themselves might excite an erythematous reaction in the absence of horse serum The results are given in table I (p. 246)

It will be seen that the areas of erythema produced by the control injections of both the normal serum and the antiserum are very nearly equal Further, the three reactions on the right arm, where each site had been injected with normal human serum, were all the same size, whether the area had been previously injected with horse serum or not The areas of erythema resulting from the injection of antiserum into the sites previously injected with horse serum were, however, about ten times as large as those

produced under the same conditions by normal serum or by each of the control injections of the two kinds of human serum into unprepared sites.

TABLE I

Means of the areas of the erythema resulting from the injection of normal human serum and antiserum into sites sensitised by horse serum 48 hours previously

Site of injection	Preparatory injection of horse serum	Area of erythema (sq. cm.)	
		Right arm (normal serum)	Left arm (anti-serum J.E.S.)
Upper . .	0.1 c.c. 1 : 20	1.78 \pm 0.26	16.15 \pm 1.42
Middle . .	0.1 c.c. 1 : 100	1.84 \pm 0.51	15.47 \pm 1.95
Lower . .	Nil	1.66 \pm 0.53	1.65 \pm 0.26

There was also a striking difference in the extent of the whealing produced in the two arms. On the right side (normal serum) the small skin elevations resulting from the injections soon subsided and at no stage did characteristic wheals appear. On the left side (antiserum) the skin elevations became progressively larger, lost the more or less rounded contour of an intradermal bleb and developed the flattened plateau-like top characteristic of a spreading wheal.

Effect of dilution of antiserum on the size of the erythematous reaction

Several experiments were made to determine the extent to which the dilution of the antisera would diminish the erythematous reaction. As before, sites were prepared by the intradermal injection of 0.1 c.c. of a 1 : 100 dilution of horse serum and twenty-four hours later antiserum in dilutions of 1 : 25, 1 : 125 and 1 : 625 was injected at the same points—antiserum from one sensitive subject (J.E.S.) into the right arm and that from the other (G.M.C.) into the left. The weakest dilution of antiserum was placed midway between the other two. The results are given in table II.

It is evident that both antisera are capable of causing reversed reactions even after considerable dilution, though, as would be expected, the size of the erythematous reaction diminishes steadily with increasing dilution. Antiserum J.E.S. had become irregular in its action, as can be seen from the relatively high value of its standard error, in the dilution 1 : 125, but antiserum G.M.C. showed considerable persistent activity even at the highest dilution (1 : 625). In each of the subjects examined the whealing was better with antiserum G.M.C., and it was unfortunate that more of this antiserum was not available for further experiments.

TABLE II

Means of areas of erythema resulting from the injection of varying dilutions of antiserum into sites sensitised by horse serum 24 hours previously

Human antiserum	Area of erythema (sq. cm.)		
	Dilution of serum		
	1:25	1:125	1:625
JES	5.45 ± 0.57	3.42 ± 1.22	2.24 ± 0.56
GMC	7.46 ± 0.94	5.56 ± 0.92	4.48 ± 0.92

Time required for sensitisation of the skin by the horse serum injected

In order to determine the time necessary for the injected horse serum to sensitise the local tissue cells, several experiments were carried out in which a series of sites, prepared simultaneously beforehand, was injected with antiserum one at a time at varying intervals. Six sites—three in each forearm—were injected intradermally with 0.1 c.c. of a 1:100 dilution of horse serum. The reactions evoked were slight and the injected fluid was completely absorbed in less than half an hour. After half an hour, one hour, two, five, twelve and twenty four hours, 0.1 c.c. of human antiserum was injected into the six sites *serialim*. After twenty minutes the reactions were recorded and the means and standard errors of the areas of the resulting flares and wheals are set out in table III. The size of reaction caused by a control injection of the antiserum into an area of normal skin at the end of the first hour is also recorded.

TABLE III

Means of areas of erythema and wheals developing when antiserum is injected at various intervals after sensitisation of skin sites by horse serum

No. of subjects used	Dilution of antiserum (JES)		Area of erythema (E) (sq. cm.) and wheal (W) (sq. mm.)						
			Time (hours)						
			½	1	2	5	12	24	Control
2	Undiluted	E	9.59	12.93	12.81	14.48	15.48	11.96	2.82
		W	102	152	218	197	278	177	83
5	1:5	E	4.98 ± 1.13	8.36 ± 1.37	8.64 ± 0.97	10.78 ± 0.98	12.83 ± 1.45	9.98 ± 1.10	1.50 ± 0.34
		W	67 ± 10	97 ± 21	130 ± 19	134 ± 16	159 ± 15	125 ± 14	37 ± 11

It can be seen from this table that a very definite reaction involving both erythema and wheal developed when the interval between the injection of the horse serum and of the antiserum was as short as half-an-hour. The reaction in the site injected at the end of one hour was considerably larger and this increase in the area of the flare continued until twelve hours. Two of the five subjects who received the diluted antiserum, however, had the largest of their reactions at the end of five hours, from which it would seem that in general the maximal reactions may be expected about the tenth hour. With an interval of twenty-four hours the size of the reactions had fallen somewhat and was much the same as had been found previously with reacting solutions of similar strengths. The wheals resembled the flares in rising to maximal mean areas at the end of twelve hours; one of the subjects, however, had his largest wheal after five hours and one at the end of twenty-four hours. Both subjects injected with undiluted antiserum had their maximal reactions, both for flares and wheals, after twelve hours, and from the findings in these two subjects it appears that the flare, at least, rises towards its maximal size rather more rapidly than when the more dilute antiserum is used. The possibility that this observation is related to the greater ability of the stronger antiserum to neutralise any excess of free antigen is discussed below.

In both series the injections of antiserum alone excited only small flares and wheals.

*Comparison of the sensitivity of the reversed reaction
with that of Prausnitz and Küstner*

In ten subjects the skin of the right forearm was injected intradermally with 0.1 c.c. of a 1:100 dilution of horse serum at three points, and at the same time the skin of the left forearm was similarly injected at three symmetrical points with 0.1 c.c. of various dilutions of human antiserum—at the highest point in the left arm undiluted, at the middle point 1:25 and at the lowest 1:5. Twenty-four hours later the injections were repeated, but the right arm was now injected with the human antiserum and the left with horse serum. A group of reversed reactions was thus produced in the right arm and of Prausnitz-Küstner reactions, using identical amounts of both antigen and antibody, in the left arm. Since both reactions were completed within twenty-four hours of the initial injection of the horse serum into the right arm, there could be no risk of any neutralisation of the antigen by antibodies actively produced in the test subjects. The results are set out in table IV.

Since the number of subjects examined was small the significance of the differences has been tested by the method devised by Fisher (1938) for assessing the significance of differences between the means of small samples. At each dilution of antiserum the significance is of a high order. These experiments seem to show, therefore, that the reversed reaction is still strongly present at levels of antigen and antibody concentration at which the Prausnitz Kustner reaction has reached the lower limit of its sensitivity. Not only did the former produce larger areas of erythema, but it also caused more definite whealing, this, indeed, was often marked on the right arm when none was present on the left.

TABLE IV

Comparison of the means of the areas of the erythema developing in reversed passive sensitisation and in the Prausnitz Kustner reaction

Dilution of serum (J.F.S.)	Area of erythema (cm ²)		
	Reverse passive sensitisation	Prausnitz Kustner reaction	Difference between means
Undiluted	13.03 \pm 1.27	2.29 \pm 0.27	10.74
1:5	10.47 \pm 1.14	2.40 \pm 0.43	7.98
1:25	9.75 \pm 1.54	2.09 \pm 0.42	7.66

There remained, however, the possibility that, in the simultaneous performance of two reactions on the two arms of the same person, some of the antigen might enter the circulating blood in one arm and be carried to the tissues of the other, where it might partly or completely neutralise the antiserum already injected there and so lessen the intensity of the Prausnitz Kustner reaction when this was elicited later. Actually, Gay and Chant (1927) found that, with ragweed pollen extracts, "contralateral injection" of antigen brought out the Prausnitz Kustner reaction almost as satisfactorily as direct injection into the sensitised area. To exclude such possibilities a series of Prausnitz Kustner reactions in a group of five subjects was compared with a series of reversed reactions in five other subjects. In both series the concentration of the reagents and the time interval were the same as in the earlier experiments. The results are set out in table V (p. 250).

All the differences between the means are highly significant as judged by Fisher's test. There can be little doubt, therefore, from the fairly good agreement between the results set out in tables IV and V that the neutralisation of the antiserum by the horse serum injected contralaterally was not responsible for the observed differences in the two types of reaction.

TABLE V

Comparison of the means of the areas of the erythema developing in reversed reactions and Prausnitz-Küstner reactions carried out in different subjects (2 groups of 5 persons each)

Dilution of serum (J.E.S.)	Area of erythema (sq. cm.)		
	Reversed passive sensitisation	Prausnitz-Küstner reaction	Difference between means
Undiluted	12.52 \pm 2.63	1.64 \pm 0.37	10.88
1 : 5	8.19 \pm 1.23	1.39 \pm 0.13	6.80
1 : 25	7.85 \pm 1.53	1.99 \pm 0.49	5.86

DISCUSSION

It seems likely from the close similarity in their mode of production and clinical course that the present reversed passive sensitisation reaction and the Prausnitz-Küstner reaction have a fundamentally similar mechanism, and that when horse serum is used as the antigen it is immaterial for the successful production of these local reactions whether the antigen or the antibody-containing human serum is injected first and therefore comes into contact first with the tissue cells. In the light of our present knowledge of reversed passive anaphylaxis this ready reversibility of the local skin sensitisation reaction is hardly surprising, especially when normal mammalian sera, with their large assortment of protein components of various sizes, are used as antigen. Indeed, a greater ease of reversibility might be expected in skin reactions than in generalised anaphylactic reactions, for in the latter both antigen and antibody are usually injected intravenously, and their access to the tissue cells, both for sensitisation and for shocking, must consequently depend upon the readiness with which they can pass through the walls of capillaries, while in the former both the reacting substances are introduced directly into the tissue fluids immediately surrounding the reacting cells. It is quite possible that the ease or difficulty with which they can pass through the capillary walls—and this in turn may be related to their molecular size and shape—may be an important factor in determining the acceptability of proteins to the tissue cells in particular animal species. For if either the antigen or the antibody reaches the tissue fluids only with difficulty, the rate of reaction of the ensuing combination of antigen with antibody, even though it eventually proceeds to completion, may be too slow to elicit a recognisable generalised anaphylactic reaction, and the protein may consequently be classed as "unacceptable" to the animal species concerned.

The likelihood that a large number of very diverse foreign proteins are acceptable to human tissue cells when brought into intimate contact with them by direct introduction into the surrounding tissue fluids would seem to be shown by the lengthy list of antigens that have been found to be capable of evoking the Prausnitz Kustner reaction. But it is perhaps equally possible that even these proteins are not acceptable to the cells of a normal animal and only become so when the tissues have been previously exposed to the appropriate antibody as in the Prausnitz Kustner reaction, which then serves as an intermediary between the cells and the protein, having affinities specific for both. The reversed reaction, which largely eliminates the factor of capillary permeability, might be a useful method for investigating in man the acceptability or otherwise of particular foreign proteins to the unsensitised cells of the dermis.

Even supposing a foreign protein to be acceptable to the cells, time must elapse before their sensitisation is complete. During this interval part of the antigen will remain free in the surrounding tissue fluids and part will be fixed to the local cells. How quickly the former part diminishes and the latter increases is not easy to determine, but the fact that the intensity of the reversed reaction with horse serum does not reach its maximum for about ten hours clearly indicates that the combined processes take place slowly. This does not mean, however, that the completion of the sensitisation of the cells necessarily takes this length of time. It may merely mean that it requires this period for the removal by capillaries and lymphatics of any excess of antigen that remains free in the tissue spaces after the cells have become saturated and is available to combine extracellularly with any antibody introduced. Such combination of antigen and antibody in the tissue fluids would not merely fail to add to the intensity of the reaction but would probably minimise it by neutralising the antibody before it reached the fixed antigen to evoke the characteristic response. The more rapid rise in the intensity of the reversed reaction when the stronger antibody solutions were used would seem to show that this extracellular neutralisation may be taking place on a relatively large scale when the time interval between the two injections is short. A comparable time interval between the injection of human antiserum and antigen (horse dander) was found necessary by Coca and Grove (1925) to secure a maximal Prausnitz Kustner reaction and it is possible that the progressively decreasing ratio of free to fixed antibody—and therefore the decreasing proportion of extracellular neutralisation of antigen—may provide a similar explanation for their observation.

The explanation of the difference in severity of the response between the reversed reaction and the Prausnitz Kustner reaction

probably lies in differences in the effective concentrations of antigen and antibody present in the injected area of skin at the time the two reactions take place. Any protein, homologous or heterologous, will escape from the injection area, largely through blood and lymph capillaries, so that its concentration round the point of injection progressively falls. If the injection of a foreign protein be made into an unsensitised animal the rate of escape will be more rapid than in a sensitised one, so that the concentration may soon fall well below that initially present. Since, as Tuft and Ramsdell (1929) found, the precipitin titre of human sera capable of transferring passive sensitivity to horse serum is often low or even undetectable, it seems quite likely that in both types of reaction studied in the present paper the concentration of antigen used, as judged by immunological equivalents, will be greater than that of antibody, irrespective of whether it be injected first or second. In their study of the passive transfer of sensitivity following serum sickness, Tuft and Ramsdell found that positive Prausnitz-Küstner reactions could be produced with as little as one-fiftieth of the quantity of horse serum used in our present experiments. Consequently, were the antigenic protein to be given 24 hours' start to escape from the site of injection, enough of it would probably still remain to react to the maximal degree with the amount of antibody introduced later. Were the antibody to be injected first, the opportunity that the interval would give for its escape would materially lower its concentration in the area later injected with antigen. If this suggestion be correct—that the local antibody concentration *at the time of its neutralisation* is the factor determining the severity of the response—the order of injection of the antigen and antibody would affect the comparative severity of the two types of reaction in the manner found in these experiments.

This relatively greater sensitivity of the reversed passive sensitisation reaction when the interval between the injections of the two reacting serum solutions was only 24 hours might well tend in the direction of lesser sensitivity if the period were extended. For if several days were to elapse between the two injections, actively formed antibodies against the horse serum might begin to appear in the circulation and diffuse into the tissue fluids of the sensitised area, to combine there with the residue of the antigen. In their studies on the sequence of a series of reactions in human skin after injection with horse serum, Kellett and Wright (1930) suggested that the neutralisation of antigen in non-sensitive subjects probably begins after several days, and depends upon the appearance after that interval of actively produced antibodies to the foreign serum. But in the interval before this specific neutralisation begins, the disappearance of the horse serum proteins from the injected site would merely take place by diffusion into the sur-

rounding tissue fluids and removal by the blood and lymph streams, and enough would remain locally to react with the appropriate antibodies should they be injected into the sensitised area and so produce a visible erythematous response.

SUMMARY

1 A local skin reaction has been produced in human subjects by the injection of serum from a person sensitive to horse serum into a skin area previously injected with horse serum.

2 The reaction was not produced when serum from persons not sensitive to horse serum was injected into similarly prepared areas.

3 The reaction could be produced even after considerable dilution of the human anti-serum used, one anti-serum giving definite results in a dilution of 1/625.

4 A reaction could be obtained when the anti-serum was injected half an hour after the horse serum but a maximal response did not develop unless an interval of several hours had elapsed.

5 Comparison under similar conditions with the Prausnitz-Kustner reaction for horse serum showed that the present reaction is more sensitive.

6 A possible explanation for this increased sensitivity is discussed.

We should like to express our thanks to our colleague Mr J. H. Spalding, for his kindness in allowing us to take samples of his blood on many occasions.

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THE ADHESIVENESS OF BLOOD PLATELETS IN NORMAL SUBJECTS WITH VARYING CON- CENTRATIONS OF ANTI-COAGULANTS

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THE mechanism of thrombus formation was first studied by Zahn (1875), who observed that collections of white cells accumulate on the wall of a blood vessel at a point of injury, or on the surface of a foreign body exposed to circulating blood. The important part played by platelets in the formation of such thrombi was first recognised by Hayem (1878) in the course of his pioneer work on these blood elements. In 1886 Eberth and Schimmelbusch, repeating some earlier work by Bizzozero (1882), showed that white thrombi were formed by the adhesion of both white cells and platelets to the damaged vessel wall at a site of injury, and ascribed the aggregation of the platelets to a "viscous metamorphosis" of these elements. This viscous or adhesive property of platelets was further discussed by Osler (1886) who pointed out (p. 378) that their "tendency to adhere to foreign particles is very noticeable". The processes involved in the formation of white thrombi by the laying down of platelets under various conditions have more recently been studied by Shionoya (1927) and Rowntree and Shionoya (1927), and by Best and his co-workers (1938-39, 1939).

From this early work it is apparent that the adhesiveness of platelets has long been recognised as one of their more obvious characteristics, yet no attempt has so far been made to study the property in any quantitative manner. On the other hand for leucocytes, which exhibit the same property, methods have been devised for measuring the stickiness by Fenn (1922-23) and Ebergényi (1934). Mention of stickiness of both leucocytes and platelets, though without any direct attempt to measure the property, has also been made by Abramson (1928) in his reports on the cataphoresis of blood cells.

The present study records measurements of the adhesive properties of platelets in the presence of varying concentrations of certain anti-coagulants. The assumption made by Baar and Székely (1929), MacKay (1930-31), Olef (1936-37), and Lee and

Erickson (1938-39) that platelets disintegrate in the blood is also discussed, and it is suggested that their apparent disappearance is due to their removal by adhesion to the glass walls of any vessel in which the sample is placed.

Method

To prevent the clotting of the blood several anti-coagulants were used in various concentrations: heparin, 0.05, 0.20 and 0.80 mg. per c.c.; potassium oxalate, 0.58, 2.32 and 4.64 mg. per c.c.; chlorazol blue, 1.2, 4.8 and 9.6 mg. per c.c.; and chlorazol pink, 0.3, 1.2 and 2.4 mg. per c.c. In every case it was desired to use the minimal amount that would prevent clotting, and to compare the extent of the removal of the platelets from this and other samples containing multiples of this minimal amount. Solutions of suitable strength of each anti-coagulant were made up and the above quantities measured into waxed beakers and dried at 37° C.

The subjects were nurses and male medical students. All were healthy and had red cell counts within the limits of the normal as determined by

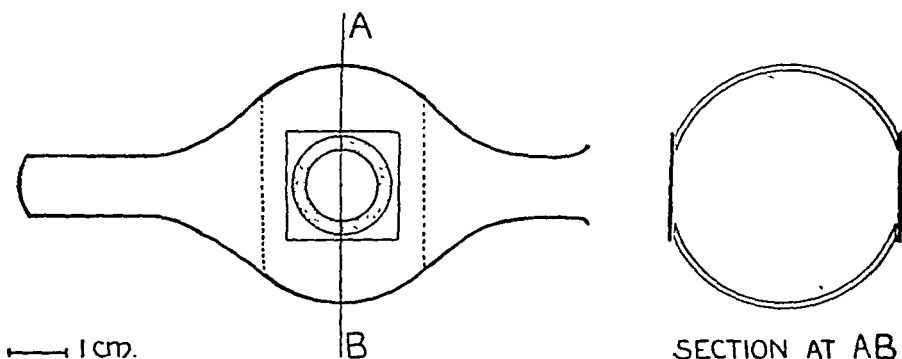


FIG. 1.—Elevation and cross section of tube used. Stippled circle is the ground glass surface to which the cover-slip is luted by vaseline. The blood circulates on a track enclosed by the dotted lines.

Price-Jones *et al.* (1935). Venous blood was withdrawn into an all-glass syringe thinly smeared throughout with liquid paraffin. Five c.c. of the blood were immediately transferred to each of the waxed beakers and agitated to ensure complete mixing with the anti-coagulant. Within 5 mins., 2 c.c. from each sample were transferred to a special glass tube with a central bulb whose sides had been carefully ground away to form open windows in two places about 1.5 cm. in diameter (fig. 1). These windows were closed by cover-glasses kept in place by vaseline. The internal edges of these windows were ground to a sharp edge to minimise any internal "shoulder" between the bulb and the cover-glass at which small quantities of blood might stagnate. As a control, 2 c.c. of blood containing the least concentration of anti-coagulant were placed in a similar tube treated with melted vaseline and drained. All tubes were rotated at $3\frac{1}{2}$ r.p.m. on a motor-driven wheel so that the blood was constantly passing over a known area of glass (about 33 sq. cm.) at a steady rate.

Every twenty minutes platelet counts were made on samples withdrawn from each tube. These were expressed as percentages of the initial count. At the end of 80 mins. the cover-glass windows were removed, dried and stained by Leishman's method.

All platelet counts were made by the direct wet method, the whole field of a Burker chamber being counted. This method was chosen as being the most suitable after considerable trial of both wet and dry, direct and indirect methods, since results were consistent and the speed of counting—a necessity in this work—was greater than with indirect counts. Rees-Ecker diluting fluid, advocated by Tocantins (1937) but modified by the addition of 2 per cent formalin instead of 0.2 per cent in order to fix the platelets immediately on their coming into contact with the fluid, was used. In order to prevent the adhesion of the platelets to the dry stem of the pipette the diluting fluid was first drawn up to the 0.5 mark and the blood then drawn into this up to the 1.0 mark. All technique was standardised as far as possible, the blood with each concentration of anti-coagulant being counted with the same pipette throughout and in the same counting cell.

Variations within the limits of normal of the initial platelet counts were ignored because the percentage number removed at 20 minute intervals from a given sample of blood was calculated on the initial count of that sample. Variations caused by stimuli such as exercise, temperature, posture etc. noted by Mackay (1930-31), Steiner and Gunn (1931), Beecher (1935-36) and Lee and Frickson (1938-39), would not influence the results of the present investigation. All subjects were about their normal hospital duties. Pohle's (1939) observation that platelet counts in women fluctuate during the menstrual cycle made it desirable to record the findings in 10 normal women separately from those of the men. Samples were taken from women at all stages of the cycle, but though the initial counts varied according to Pohle's observations, no significant effect on the stickiness of the platelets was detected and the average findings in the 10 females closely resembled those of the males (fig. 2).

Results

All the subjects investigated had red cell counts between 4.87 and 5.72 million per cmm and were regarded as being normal. The mean of the platelet counts, with its standard error, was 323 ± 18 thousand per cmm. All counts fell within twice the standard deviation from the mean.

The anti-coagulant substances used prevented clotting of all blood samples of which data are recorded, but difficulty was often experienced with the chlorazol dyes. With both chlorazol fast pink B K S and chlorazol sky blue F F a thin film of coagulum formed on the surface of the glass when the concentrations recommended by Huggett and Silman (1932) were used. Higher concentrations (1.5 times the former figure) were therefore adopted but even then a deposit often formed and rendered the counting of platelets impossible. This deposit appeared to be amorphous and contained neither platelets nor red cells. By carrying out many experiments, however, and excluding all those in which deposits were formed, it was possible to procure three (one with chlorazol pink and two with chlorazol blue) which were suitable for comparison with the other anti-coagulants used.

The average rates of fall of the platelet counts in the blood samples in the rotating tubes, expressed as percentages of the initial

counts, are set out in figs. 2 and 3. By difference, this shows the average percentage of platelets removed from the blood in every twenty-minute period of the experiment and the corresponding figures for the vaselined control tubes.

Similar results were obtained with each of the anti-coagulants used. In all cases there was a progressive fall in the platelet content of the blood along curves of logarithmic type and the rate of fall was dependent upon the concentration of the anti-coagulant. With

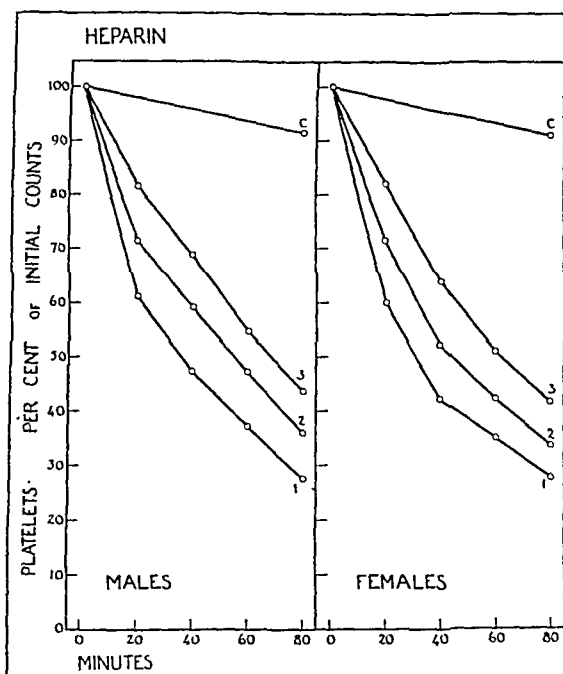


Fig. 2.—Decrease in platelets expressed as percentage of initial counts. Average of 10 cases.

Curve C = control.

„ 1 = lowest concentration anti-coagulant.

„ 2 = intermediate concentration.

„ 3 = highest concentration.

minimal concentrations, *i.e.* with just sufficient anti-coagulant to prevent clotting, the final average counts (curves 1) after 80 minutes were between 22 and 28 per cent. of the initial counts. With the highest concentrations (curves 3), however, the variations in the rate of fall with the three anti-coagulants were more marked. With heparin (16 times the minimal concentration) the final average count after 80 minutes was about 43 per cent., while with sodium oxalate (8 times the minimal concentration) it was only about 58 per cent. of the initial counts. With the chlorazol dyes the counts dropped at an intermediate rate. When 4 times the minimal

concentration of anti coagulant was used (curves 2) the rate of fall in all cases was intermediate between those found for the highest and lowest concentrations. It appears, therefore, that the greater the concentration of the anti coagulant the less the stickiness of the platelets and that this inverse relationship is present irrespective of the anti-coagulant used. In all the control tubes the results were approximately the same, the diminution in platelet counts being very much less in all cases (curves C).

The adhesion of the platelets to the glass was demonstrated by staining the cover glass windows at the end of each experiment

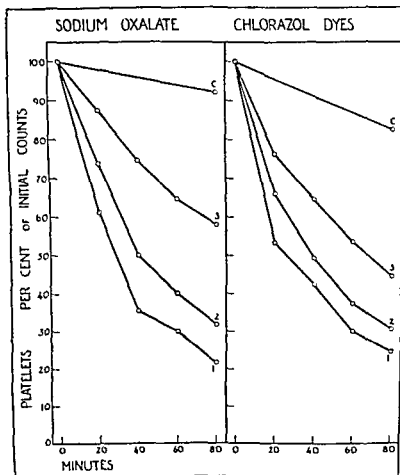


FIG 3—Decrease in platelets expressed as percentage of initial counts. Oxalate, average of 10 cases. Chlorazol dyes, average of 3 cases. Curves as in fig 1.

Large plaques of platelets were present all over the window with minimal concentrations of anti coagulant. These plaques consisted of uncountable numbers of platelets stuck together in several layers, so that often they occupied the entire field of the oil immersion lens. With the highest concentrations of anti coagulant plaque formation was less marked, the platelets tending to lie singly as though adherent to the glass rather than to each other.

It was not possible to stain the windows of the control tubes, but, as the counts diminished by so little during the experimental period, it may be assumed that comparatively few had adhered to the vaselined surface.

Discussion

Most workers who have counted the platelets at varying intervals in drawn blood have found that their number progressively decreases. Baar and Székely (1929), who made some of the earliest observations on their disappearance, kept samples of blood in hæmatocytometer pipettes and made counts at intervals from 20 to 80 hours. They assumed that the fall in number was due to disintegration, but they failed to examine the internal surfaces of their pipettes for the presence of the missing platelets. The same criticism may be directed against the work of MacKay (1930-31). Olef (1934-35, 1936-37), Tocantins (1937, 1938). Walker and Sweeney (1939-40) and Lee and Erickson (1938-39), who also failed to examine the walls of their containers, although it is a well-established observation that platelets adhere readily to glass.

Ferguson (1934), using dark ground illumination, studied the sequence of changes in platelets in contact with glass surfaces. He described the following changes, which take many hours for their completion: (1) swelling, (2) adherence to glass, (3) "spreader forms", (4) formation of processes, (5) bursting of processes, (6) final loss of cell outline. He also noted that this sequence took place more slowly in citrated blood. He specifically stated that the terms "disintegrative" and "necrobiotic" were inappropriate.

The type of chamber used in the present study was designed to enable the wall of the glass vessel to be readily examined at the end of the experiment. Throughout this period the cover-glass windows were in contact with the blood to the same extent as other parts of the wall and the presence upon them of enormous numbers of platelets proves that much of the reduction in the counts results from the adhesion of these cells to the glass wall. Thus the view that the fall in numbers is due to disintegration must be modified. The slight fall in platelet counts in the control tubes may be due to a small proportion being disintegrated, but as no method of staining the windows of these tubes was found, it is quite possible that a part or even the whole of this small percentage may have adhered to irregularities in the vaselined surface.

A further reason for rejecting the view that extensive platelet disintegration takes place *in vitro* is that the large quantities of thrombokinase thus liberated would, in those samples containing the minimal quantities of heparin or chlorazol dyes, exceed the capacity of the anti-coagulant to prevent clotting. No such clotting was observed in these samples.

The mode of action of the anti-coagulant, whether anti-prothrombin or anti-calcium, appears to have little effect upon the rate of removal of the platelets. The percentage removed is dependent rather upon the concentration of the anti-coagulant

than upon its mode of action. It seems likely, therefore that the degree of stickiness of the platelets is affected directly by the failure of the chemical changes incipient in clotting. This finding is of interest in the light of the suggestion made by Chargaff *et al* (1936) and of Best's (1938-39, 1939) subsequent work on thrombus formation. These authors believe that heparin or a heparin like substance occurs in the general circulation and acts as an anti-prothrombin, neutralised by thrombokinase. It is probable, therefore, that if heparin acts directly upon thrombokinase it will operate least successfully at the surface of the platelets where this substance is released and is in the highest concentration.

In studying the effect of heparin on thrombosis Shionoya (1927) and later Best (1938-39) and Best and Campbell (1938) found that with higher concentrations of this anti coagulant thrombus formation was retarded though not entirely prevented. Further work by Best *et al* (1938, p. 28) showed that this was true only of rabbit's blood and suggested that rabbit platelets are either "more easily destroyed" or "liberate a more powerful 'adhesive agent'" than those of dogs, cats and monkeys. In this respect human platelets may resemble those of the rabbit.

The similar retardation in the adhesion of platelets to the walls of the glass containers in the presence of oxalate may be due to a failure in fibrin formation following precipitation of the ionised calcium. The degree to which this may be checked may depend on the amount of oxalate present, up to a maximum when all the calcium is precipitated.

The stickiness of platelets may be due to the formation on their surface of a thin film of fibrin (Shionoya) and should this be the case it explains the variations observed with the different concentrations of anti coagulant. Thus in blood samples having a high concentration of anti coagulant any thrombokinase liberated from the platelet surface is relatively ineffective in forming fibrin either because of direct neutralisation in the presence of heparin and the chlorazol dyes or of the absence of calcium ions in the presence of oxalate. With minimal concentrations of anti coagulant this neutralisation may be incomplete, so that the presence of a thin film of fibrin renders the platelets sticky. Further work now proceeding on pathological and post operative conditions may help to throw more light upon the source of the adhesive property of platelets.

Summary

1. A method of measuring the adhesiveness of platelets is described.

2. The effects on the stickiness of platelets of the anti-coagulants heparin, sodium oxalate and two chlorazol dyes in various concentrations have been measured.

3. The greater the concentration of these anti-coagulants, the less is the stickiness of the platelets.

4. The progressive fall in platelet counts of blood *in vitro* is shown to be mainly if not entirely due to their adhesion to the glass walls of the container.

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GIANT-CELL CHRONIC ARTERITIS

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(PLATES XVII AND XVIII)

FOUR cases are described of a rare and not generally recognised form of arteritis which affects the aorta the branches of the aortic arch and their branches, and possibly other arteries

Case 1

Clinical history

The patient, a woman of 23, had had diphtheria at 3 and acute articular rheumatism at 17. Six months before death she developed attacks of giddiness and hot sweats, lasting about 10 minutes and occurring two or three times a day every three weeks just before or just after a period. Eight weeks before death a lump in the neck was noticed which became larger. Five weeks before death she had pain in the right arm beginning in the shoulder and spreading down to the hand, the arm becoming numb and weak. On admission two weeks before death, she was pale and slightly wasted. There was slight pitting œdema of the ankles. A few slightly enlarged cervical lymph glands were present on both sides, one of which, from the right side, showed miliary tuberculosis. A lump c. 14 x 1 in. in the right supraclavicular fossa, pulsed with the cardiac systole and there was a systolic thrill on its outer side. The apex beat was forcible and half an inch outside the mid clavicular line in the fifth intercostal space. A loud systolic murmur replaced the first sound at the aortic area and was conducted into the neck towards the supraclavicular lump. Forcible pulsation was present in the supra-sternal notch and over the abdominal aorta and femoral arteries. The pulse rate was 92. The right radial artery was tortuous and its pulse regular, hard and of small volume. The left radial pulse was almost imperceptible. The blood pressure (right arm) was 160/130 mm Hg on one occasion, and 220/130 on another. In radiographs the aorta, trachea and œsophagus were displaced to the left and a shadow was present over the apex of the right lung. The left pupil was larger than the right. There was weakness of all movements of the right arm and analgesia over the distribution of the first right thoracic nerve. There was no pyrexia. She had a sudden hæmoptysis of about 5 oz of bright red frothy blood and died shortly afterwards.

Post mortem findings

Summary of necropsy Chronic arteritis of aorta and branches of its arch. Rupture of aneurysm of right subclavian artery into pleural cavity and lung. Cardiac hypertrophy (hyperpiesia)

Miliary tuberculosis of upper cervical lymph glands. Negative Wassermann, Kahn and Laughlen reactions upon post-mortem blood.

Macroscopic appearances. Left ventricle moderately hypertrophied. Distal half of aortic arch and descending thoracic and abdominal aorta very slightly dilated (up to 7 cm. in circumference) and thickened (0.4 cm. thick), their walls white, fibrotic and inelastic. These parts showed abnormally severe atheroma and much longitudinal wrinkling of intima. There were two saccular aneurysms (1 cm. in diameter) on the distal half of the arch. Abdominal aorta adherent to lumbar spine. Great narrowing of lumen by white intimal thickening in a 3 cm. segment of left subclavian artery, beginning 0.5 cm. beyond its origin. Proximal 4 cm. of right subclavian dilated to 4.5 cm. in circumference; wall thickened and rigid and showing severe atheroma. Immediately distal to this there was an aneurysm (6 × 5 cm.) which communicated with the artery by an orifice 10 × 3 mm. Wall of aneurysm up to 1.5 cm. thick, the outer part fibrous, the inner blood clot. Aneurysm situated behind the right clavicle; the upper border of the first rib projected into it. Its lower part was ruptured (1.5 × 0.5 cm.) into the pleural cavity. Walls of upper 1 cm. of right common carotid artery and lower 1 cm. of right internal carotid thickened, fibrotic and rigid and showing much atheroma. Orifice and proximal 0.5 cm. of right external carotid artery occluded by white intimal thickening. Lower 1 cm. of left internal carotid artery and upper 1.5 cm. of left common carotid showed similar changes. Lumen of beginning of left external carotid greatly stenosed (down to 2 mm. in diameter) by white intimal thickening. Remainder of common, internal and external carotid arteries, ascending aorta, proximal half of aortic arch and pulmonary arteries normal.

Clotted and fluid blood (1½ pints) in right pleural cavity. Rupture (2.5 cm. long) in visceral pleura of upper lobe of right lung opposite rupture of aneurysm. Lung tissue beneath ruptured up to 3 cm. deep. Extensive solid hæmorrhage in right upper lobe around rupture and numerous small peribronchial hæmorrhages throughout remainder of lungs. Subpleural hæmatoma (7 × 5 cm.) in anterior part of right upper lobe. Left pleural cavity largely obliterated by fibrous adhesions. Body well nourished and condition of teeth good.

Microscopic appearances. In the abdominal and descending thoracic aorta muscle fibres and internal and external elastic lamellæ were absent from the media. The elastic fibres were usually well preserved but some were swollen or fragmented. In an area near one end of the section of the abdominal aorta the media was up to 812 μ thick and the elastic fibres were widely separated by proliferated fibroblasts, a little collagen and a few lymphocytes, plasmacytoid

In a few areas in the stenosed segment of the *left subclavian artery* the media was slightly thinned and the internal elastic lamella and muscle fibres were absent so that the elastic fibres had collapsed together. In such abnormal areas a few vessels surrounded by fibrous tissue disorganised the elastic. In one place there were a few lymphocytes. The adventitia showed slight fibrous thickening and infiltrating lymphocytes. The intima was greatly thickened so that the lumen was reduced to a small slit. It contained numerous muscle fibres and delicate elastic fibres and was fibrotic in places. A new internal elastic lamella had been formed around the lumen, but it was separated from the endothelium by a layer of muscle fibres and delicate elastic and collagen fibres.

The beginning of the *right external carotid artery* showed a normal media except for slight fibrosis and slight focal loss of elastic from the outer part. The lumen, however, was occluded by a great intimal thickening consisting of very delicate collagen and elastic fibres and numerous muscle cells in an oedematous ground substance.

Case 2

Clinical history

The patient, a woman of 59, suffered from "influenza" five months before death and took to bed. A month later she had almost recovered but began to suffer from severe pain shooting from the left ear up the left side of the head and she had "noises like an engine" in the ear. Nine weeks before death the pain became worse and vomiting occurred and became persistent. She again took to bed. A week later she developed a cough and a rash of raised red spots on the body, diagnosed as measles, which faded in two weeks. She lost weight and noticed sweating of the head. On admission, four weeks before death, she was pale, thin and sweating. The skin of the hands and feet was peeling in large flakes. The few remaining teeth showed severe chronic periodontitis with much tartar and pocket formation. Eight days before death she developed a flaccid left hemiplegia and passed into a coma from which she did not recover.

Post-mortem findings

Summary of necropsy. Chronic arteritis of intracranial internal carotid arteries and aorta. Thrombosis of internal carotid and cerebral arteries. Cerebral infarction. Infarct and embolus in lung. Scars of healed gastric ulcers. Atrophy of thyroid. Calcareous nodule in bronchial gland.

Macroscopic appearances. Tough yellowish-white thickening of wall and great reduction of lumen of a 7 mm. segment of each internal carotid artery beginning at upper end of intra-osseous part and ending at bifurcation. Lumen in these segments occluded by thrombus. Left middle cerebral artery and small cerebral arteries on convexity of brain also occluded by thrombus. Intima of ascending aorta and arch showed slight gelatinous thickening.

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FIG. 1.—Case 1. Abdominal aorta. Limits of media defined by ink lines. Area of chronic inflammation of media containing a few giant cells. Focal necrosis of inflamed media on right. Fibrous thickening of intima above, with slight chronic inflammation in its outer part. Fibrosis and cellular infiltration in adventitia. Weigert's iron hematoxylin and Ponceau S, picric acid mixture. $\times 70$.

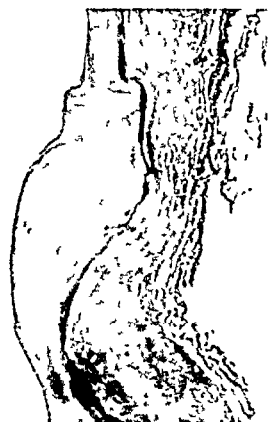


FIG. 2.—Case 1. Descending thoracic aorta. Media represented by a layer of elastic fibers with the known giant cells within them. In adventitia an hyaline, the latter containing an alternating layer. Part is elastic stain and Ponceau S, picric acid mixture. $\times 70$.

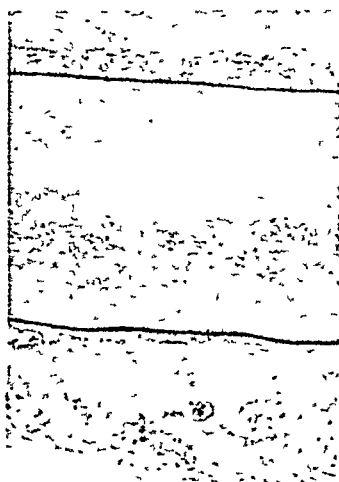


FIG. 3.—Case 2. Ascending thoracic aorta. Limits of media defined by ink lines. Infiltration of media and outer part of hyperplastic intima. Slight thickening of adventitia. Hematoxylin and eosin. $\times 70$.

Considerable amount of atheroma in aorta, but probably not excessive for age. White matter of brain friable and grey matter had a diffuse pink colour. Infarct in lower lobe of right lung and a small thrombotic embolus in an artery in middle of right lung. Small calcareous nodule in a bronchial gland. Liver diffusely congested. Great fibrotic atrophy of thyroid gland. Two scars of healed gastric ulcers. Body considerably wasted.

Microscopic appearances. The ascending and descending thoracic and abdominal aorta showed absence of the internal elastic lamella and very slight fibrosis in places in the media, which was slightly thinned in one place. These were probably changes of chronic medial degeneration. There was patchy infiltration of the media with lymphocytes and a few plasma cells, most marked in the abdominal aorta and least in the ascending thoracic (fig. 2). In the latter there were also a few neutrophil leucocytes. The infiltration was most marked in or limited to the middle of the media. In infiltrated areas the muscle fibres had disappeared and the elastic fibres had become widely separated. Where the infiltration was most marked there were a few capillaries and a few proliferated fibroblasts with, in the abdominal aorta, a little collagen formation. The adventitia showed fibrosis and slight thickening and there were lymphocytes and a few plasma cells around the vessels. The intima was considerably thickened. In the ascending thoracic aorta it consisted of numerous muscle cells, some of which were hypertrophied, a scanty amount of collagen, an abundant mucoid oedematous matrix and no elastic. In the other arteries the outer part of the intima was very fibrotic and contained a moderate number of elastic but few muscle fibres, while in the inner part there were numerous muscle fibres but no fibrosis or elastic. In the ascending thoracic aorta there were a few lymphocytes and plasma cells in places in the outer part of the intima (fig. 3). Frozen sections stained with Scharlach R showed a very little extracellular granular fat in the intima of the ascending and descending thoracic aorta and much in the abdominal aorta, but no atheromatous deposits. No giant cells were seen in these arteries.

In the thickened segments of the internal carotid arteries the internal elastic lamella was crenated, fragmented and focally absent or calcified. This change was probably due to chronic medial degeneration. The media showed marked loss or complete absence of muscle fibres, with replacement by a very delicate granulation tissue consisting of a few fibroblasts and capillaries and a very little collagen. It was densely infiltrated with lymphocytes and a few plasma cell and multinuclear giant cells (fig. 4). The giant cells were usually of foreign body type but one was of Langerhans type. Most were situated near the internal lamella and two enclosed fragments of elastic. The

showed fibrous thickening and foci of plasma cells. The outer part was fibrotic and contained a little lymphocytes and plasma cells. The wall with very little collagen and no elastin. The lumen was occupied by thrombus. The artery near its junction with the circle of Willis, about half the circumference, showed thickening of the media, slight fibrosis of the adventitia and thickening (fig. 5). There were a few plasma cells in the media and adjacent intima. The lumen was occupied by thrombus.

Case 3

Clinical history

The patient, a man of 63, had suffered from increasing weakness, loss of weight and mental inertia before death and since then had had occipital headache. Before death he developed pain in the back of the neck when standing, vomiting and vertigo. Thirteen days before death he developed tinnitus in both ears and four weeks later he was weak and felt dead. Shortly afterwards vision began to fail. Six weeks before death, he was slightly wasted and on neurological examination showed complete left homonymous hemianopia, convergence of both eyes, deviation outwards of the right eye, left proptosis, inequality of the pupils, slight left facial weakness of the tongue to the right, absence of abdominal reflexes, loss of joint sense and astereognosis of left hand, loss of joint sense in the fingers of the left hand, weakness of the grip and abduction of the fingers of the left hand. Reaction negative in blood and cerebrospinal fluid. Total protein 360 mg. per cent. of protein and 650 white cells per mm., with 10% of lymphocytes. Blood pressure normal. While in hospital no convulsions occurred. A deep-seated lesion in the right cerebral hemisphere was diagnosed. Thorotrast was injected into the right internal carotid artery for an arteriogram and the skull trephined for ventriculography. No thorotrast was seen in the right internal carotid artery, but present in the right external carotid and other cervical arteries. The patient died a few days later.

Post-mortem findings

Summary of necropsy. Chronic arteritis of segments of cranial internal carotid arteries and left external carotid artery and of three areas in aorta. Thrombosis of internal carotid artery. Cerebral infarction. Hemorrhages in pons and mid-brain. Infarcts in anterior lobe of pituitary. Calcareous nodules in lung, paratracheal and lumbar lymph glands and liver.

Macroscopic appearances. Partly fibrous, partly atheromatous intimal plaque in anterior sinus of Valsalva; two similar plaques in aortic arch. Walls of both internal carotid arteries in neighborhood of anterior clinoid processes pearly white and thickened.

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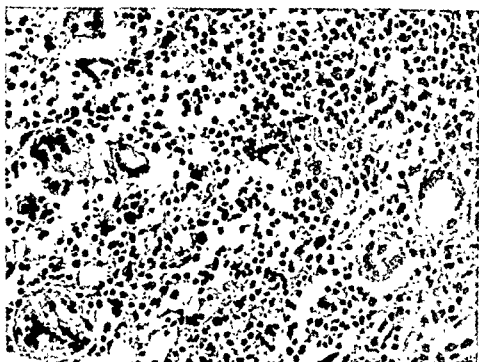


FIG. 4.—Case 2. Right internal carotid artery. Granulation tissue replacing media and containing numerous lymphocytes and some multinuclear giant cells. Hæmatoxylin and eosin. $\times 250$.

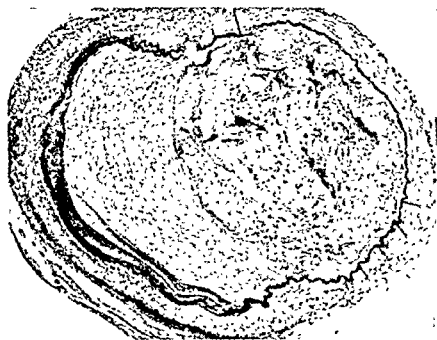


FIG. 5.—Case 2. Left internal carotid artery near circle of Willis. Large plaque of great intimal hypertrophy overlying area of inflammation in media and adventitia. Thrombosis occluding lumen. Weigert's iron hæmatoxylin, Hart's elastic stain and Ponceau S-picric acid mixture. $\times 20.5$.

to 2.5 mm, lumina greatly narrowed and occupied by ante mortem thrombus. Thrombus occluded right internal carotid artery in neck and its cavernous portion. Rupture of intima and a little ante mortem thrombus in right common carotid artery near its bifurcation at site of temporary ligature during injection of thorotrast. Numerous areas of haemorrhage throughout pons and mid brain. Area 2 cm in diameter of infarction of mesial aspect of right occipital lobe. Calcareous nodules in middle lobe of right lung, in gland near bifurcation of trachea and in liver and two in lumbar lymph glands. Fibrous adhesions obliterated right pleural cavity. Scar of healed ulcer in duodenum. No teeth present.

Microscopic appearances The thickened parts of the *intracranial internal carotid arteries* showed identical changes to those in case 2. In one place the inflammation in the media was seen to extend into the adventitia through a defect in the external elastic lamella. The *cavernous parts of the internal carotid arteries* just proximal to their thickened segments showed swelling and calcification of the internal elastic lamella atrophy of the media due to loss of some muscle cells and moderate intimal hypertrophy—changes of chronic medial degeneration. The lumen of the right artery was occluded by thrombus. In two sections of *cervical part of right internal carotid artery and one of left* there was chronic medial degeneration with intimal hypertrophy and no evidence of chronic inflammation. Right internal carotid thrombosed and in *one section taken at site of injection of the thorotrast* there was a rupture through approximately the inner half of the hypertrophied intima from this blood dissected the intima for about half its circumference. The rupture of the intima was probably the result of the injection. The accumulation of blood in the dissected intima caused considerable stenosis of the lumen. This would account for the thrombosis of the right internal carotid artery since there would have been a column of still blood between the thickened segment of the intracranial portion and the site of injection in the cervical portion. The thrombus was of the red non-laminated type as occurs in still blood. The adventitia and tissue surrounding the cervical portion of the right internal carotid artery showed oedema, haemorrhage and infiltration with large numbers of macrophages containing thorotrast. There was a little necrosis of the affected tissue. In the *right common carotid artery* at the site of temporary ligature there was chronic medial degeneration and intimal hypertrophy but no inflammation. Media and intima ruptured and media on either side of rupture necrosed and haemorrhagic. In places in the *left external carotid artery* the media was completely replaced by inflamed granulation tissue consisting of many lymphocytes and plasma cells a few multinuclear giant cells, fibroblasts and capillaries, and a very little

collagen. The internal elastic lamella was absent over these areas except for a few calcified fragments. A few of the giant cells were of Langhans type, and one other contained a fragment of elastic. Adjacent to the inflamed parts of the media the adventitia showed slight fibrous thickening and infiltration with lymphocytes and plasma cells and the intima was considerably thickened and consisted of numerous muscle cells in a mucoid ground substance with very scanty collagen and no elastic. The inflammation of the media extended in places into the intima for a short distance. At the site of the intimal plaque in the aortic *sinus of Valsalva* the media in general showed slight atrophy, fibrosis and mucous infiltration and absence of the internal elastic lamella—changes of chronic medial degeneration. In one place the media was greatly atrophied and contained a few vessels surrounded by lymphocytes and some finely granular isotropic extracellular fat and anisotropic intracellular fat. The adventitia showed slight fibrous thickening and slight perivascular lymphocytic infiltration beneath and to the side of the area of marked medial atrophy. There was great fibrosed and atheromatous intimal thickening over the area of great medial atrophy and much less intimal thickening elsewhere. Similar changes were present at the sites of each of the two intimal plaques in the aortic arch. The outer part of the intima in the plaques showed slight lymphocytic infiltration. The greatly atrophied areas in the media appeared to be focal inflammatory lesions which had almost healed by the time of death. The fibrosis of the adventitia and the lymphocytic infiltration around vessels in the media and adventitia, and in the intima in one place, give the chief support for this supposition. The anterior lobe of the pituitary showed small areas of infarction.

Case 4

Clinical history

The patient was a woman of 64. Between the ages of 14 and 21 she had suffered from an anemia which was treated with iron. During the last 30 years she had had attacks of indigestion. At 39 she was told that she had a floating kidney, and at the age of 50 she had an attack of left facial palsy which lasted a month. During the last 7 years she had suffered from arthritis in the left knee. Twenty-six months before death she developed "influenza" and since then she had lost weight and suffered from lassitude and weakness. Twelve months before death she began to have swelling of the ankles, especially at night, and a month later had an attack of abdominal pain lasting a week. She was admitted eight months before death. A mass the size of a tangerine orange was felt in the epigastrium. Bilateral corneal opacities, arthritis of left knee, varicose veins of legs and oedema of ankles were also observed. Blood pressure 210/100 mm. Hg. Urine contained one-third volume of albumin. Serum Wassermann and Kahn reactions negative. A blood-count showed erythrocytes 3,500,000; hæmoglobin 72 per cent.; colour index 1; white cells 4000 per c.mm., with neutrophil

leucocytes 19, small lymphocytes 50, large lymphocytes 25 and large hyaline cells 3 per cent. At laparotomy the abdominal aorta appeared to be dilated. She was discharged shortly afterwards. There had been no pyrexia. Seven weeks before death she developed discomfort in the abdomen, vaginal bleeding and frequency of micturition. A week before death she was re-admitted. The lower part of the distended abdomen was tender and the thighs and abdominal wall were oedematous. Per rectum a mass was palpated in the pouch of Douglas, and on vaginal examination a polyp was found in the dilated cervical canal. The urine contained one half volume of albumin. Blood pressure 130/90 mm Hg. A diagnosis of subacute intestinal obstruction was made and at laparotomy the transverse colon was adherent to the under surface of the old scar, a band constricting the left half of the transverse colon was divided. After operation the volume of urine diminished considerably and oedema of the legs and arms appeared. Death occurred three days later.

Post mortem findings

Summary of necropsy Almost healed chronic arteritis of aorta, innominate and subclavian, common carotid and common iliac arteries. Amyloidosis of kidneys and spleen. Acute enteritis. Cystitis. Adenomata in uterus. Calcareous nodules in bronchial and paratracheal lymph glands. Pleural effusions and widespread subcutaneous oedema.

Macroscopic appearances Diffuse pearly white thickening of intima of whole of aorta and innominate artery and proximal parts of subclavian arteries, less marked of common iliac and common carotid arteries. The walls of these arteries showed reduced elasticity and an amount of atheroma moderate for this age. No sign of an aneurysm of the abdominal aorta. Fibrotic nodule 3 mm in diameter, in upper lobe of left lung and two in right upper lobe. Fibrous pleural adhesions over anterior parts of upper lobes of oedematous lungs, and pleural effusions (1 pint each). Calcareous nodule in a right upper bronchial and a right lower paratracheal lymph gland. Liver congested. Spleen and kidneys oedematous looking, congested, firm and slightly enlarged. White adenoma 7×3 mm, in the subcapsular surface of the right kidney. Four endometrial adenomata, largest 2×1 cm and an adenomatous cervical polyp, 1.5×0.2 cm, in the uterus. A few patches of membrane on mucosa of injected bladder. Small intestine very friable and oedematous and mucosa in places opaque and necrotic. Lumen of appendix obliterated. Upper and lower limbs, labia and lumbar regions very oedematous. No teeth present. Body wasted.

Microscopic appearances In the descending thoracic and abdominal aorta the media was everywhere thinner than normal and averaged about 750 μ in thickness. In many irregular anastomosing areas especially in the central part, the media was interrupted by small vessels surrounded by collagen and a few lymphocytes and plasma cells. Extending from these areas into the surrounding media there were extensive patches of fibrosis in

which the elastic fibres were usually well preserved but the muscle fibres usually absent. The adventitia, thickened and fibrotic, averaged about $750\ \mu$ in thickness. It contained perivascular groups of lymphocytes. The intima was considerably thickened, averaging about $1125\ \mu$ in thickness. It consisted chiefly of dense hyaline collagen. The outermost part contained elastic and longitudinal muscle fibres, and in the remainder there were scattered circular muscle fibres and no elastic. The collagen of the intima in a few small places was impregnated with granular calcium. A small area of atheroma was present in one artery—the descending thoracic aorta.

The kidneys showed severe amyloid infiltration, chiefly in the glomeruli, which were almost entirely replaced by amyloid deposits. In the spleen there was slight diffuse amyloid infiltration of the pulp. In the ileum the mucosa was necrosed and infiltrated with bacteria and the submucosa was acutely inflamed.

DISCUSSION

There is described in the literature a rare disease called temporal arteritis which is undoubtedly similar to the lesion in my cases.

There have been 18 cases reported up to and including those of Bowers (1940) and Dick and Freeman (1940). The subjects were over the age of 54. A few weeks before the appearance of the arteritis there were usually prodromal symptoms such as headache, fever, night sweats, loss of appetite and weakness. The temporal arteries, especially their anterior branches, then became inflamed, either both about the same time or one after the other. They were enlarged, tortuous and tender and pulsation was usually absent. The enlargement was either diffuse or nodular. The pre- or post-auricular glands were enlarged in the cases of MacDonald and Moser (1936-37) and Bowers. Fever was often absent when the arteritis was present. After several weeks the arteritis subsided and the patients recovered completely. The whole illness lasted not more than six months. Segments of the affected arteries were removed in several cases. The lesion described was a chronic inflammation which affected the media chiefly and was characterised by infiltration with lymphocytes, plasma cells and multinuclear giant cells. In the cases of Jennings (1938) and Bowers, neutrophil leucocytes were also present. The intima was thickened, leading to great narrowing of the lumen which was occupied by thrombus. In Bowers's case the occipital arteries were affected besides the temporal. In Jennings's case there were signs of occlusion of the central retinal arteries. In a case of Horton and Magath's (1937) a small branch of the left radial artery was said to be also affected.

The only examples I found in the literature of an aortitis resembling that in my cases were two described by Sproul and Hawthorne (1937). The subjects were males aged 50 and 76: they died from causes other than the aortitis, which was not suspected clinically. There was no mention of prodromal symptoms. The aorta was not dilated and showed no definite abnormality except a loss of elasticity. The media was diffusely infiltrated with lymphocytes, less numerous plasma cells and multinuclear giant cells and, in one case, a few neutrophil leucocytes. Some of the giant cells

enclosed fragments of elastic. The iliac arteries were involved in both cases and the carotid arteries in one. The medial changes appeared to be almost identical with those in my case 2, except that giant cells were absent from the latter.

Bainard (1935), in a woman of 63 who died of bronchopneumonia, found a chronic arteritis affecting the coronary arteries and the cavernous and cervical parts of the internal carotid arteries. He regarded the condition as tuberculous because of the presence of endothelioid cells, multinuclear giant cells and areas of necrosis in the granulation tissue replacing the walls of the vessels. Tubercle bacilli could not be found in sections nor by the antiformin method. It is probable that this was the same type of arteritis as in my cases.

From these few reported cases and from my own, the essential features of the disease may be summarised as follows. All subjects have been 50 or more years old, except my case 1, a woman of 23. In nearly all cases prodromal symptoms suggesting an infection had been observed some weeks or months before symptoms resulted from the arteritis. In three of my cases influenza had been diagnosed. The disease has appeared to be self limited, healing after a few weeks or months. The total duration of symptoms has been at the most nine months, except in my case 4, in which 26 months elapsed between the onset of prodromal symptoms and death from amyloid disease. The arteritis, however, had subsided by the time of death. In fatal cases death was either from the indirect effects of the arteritis, such as cerebral infarction, rupture of an aneurysm and amyloid disease, or from independent causes. The disease chiefly affected the aorta, the branches of its arch and their branches. The arteries affected were as follows: temporal arteries (18 cases), aorta (6 cases), internal carotid arteries (4 cases), common carotid and iliac arteries (3 cases each), subclavian and external carotid arteries (2 cases each), and innominate, coronary and occipital arteries (1 case each). In one case the central retinal arteries were occluded, probably from the disease in more proximal vessels, and in another a branch of a radial artery appeared to be involved. In subjects with temporal arteritis the disease may have been present in the aorta and larger branches of its arch without producing symptoms. In the aorta the inflammation was widespread in 5 cases and apparently limited to three small areas in the other. In the branches of the aortic arch and their branches, the arteritis was usually focal, bilateral and symmetrical. This was well shown in the temporal arteries and in 3 of my cases in the carotid arteries.

The *macroscopic appearances* varied according to the size of the affected arteries. In the aorta in two reported cases the only change noticed was loss of elasticity. In my case 3 three prominent fibro atheromatous plaques, in case 2 a gelatinous intimal thickening and in case 4 a diffuse white fibrous intimal thickening and loss of elasticity were the only unusual changes in the aorta. In case 1,

however, the aorta showed slight dilatation, with two small saccular aneurysms, marked fibrous thickening, abnormally severe atheroma, intimal wrinkling and loss of elasticity. In the large branches of the aortic arch in this case similar changes were present, complicated in one branch by a false aneurysm. The common iliac, common carotid, innominate and subclavian arteries in case 4 showed white fibrous intimal thickening similar to that in the aorta. Smaller arteries such as the intracranial, internal carotids and external carotids showed white thickening which was chiefly intimal, and great reduction of the lumen, which was usually occupied by thrombus. In case 1 one subclavian artery was almost obliterated by intimal thickening while the other was dilated.

The *microscopic appearances* differed in different arteries of the same cases and in the same arteries in different cases. The inflammation affected chiefly the media. That in the intima and adventitia was probably an extension from the media. The inflammation in the *media* appeared to begin as a diffuse infiltration with lymphocytes and a few plasma cells and, in a few cases, neutrophil leucocytes. In the aorta it was in some cases most marked in the central part of the media. Then slight capillary invasion, fibroblastic proliferation and collagen formation appeared to follow. This was associated in most arteries with the formation of multinuclear giant cells in relatively scanty numbers. In the aorta the infiltrating cells replaced the muscle cells and widely separated the elastic fibres, while in the muscular arteries such as the intracranial part of the internal carotid the infiltrating cells lay between such muscle cells as remained or completely replaced the media. In one place in the media in case 1 there was a minute focus of necrosis of this granulation tissue, as described by Barnard in the coronary arteries in his case. In case 1 the inflammation in the media had subsided except in a few small areas. The media was thinned from loss of cells and collapse of the elastic fibres; it was composed almost entirely of elastic. Whether or not the inflammatory tissue had undergone frank necrosis before resorption could not be decided. In the aorta in case 3 the focal inflammation had also subsided, leaving foci of marked medial atrophy. In case 4 the inflammation had also subsided. There were patches of fibrosis replacing the muscle cells and areas where the thinned media was interrupted by vessels surrounded by collagen, lymphocytes and plasma cells. In the media, except in case 4, whether the inflammation was active or had subsided, there was never more than very slight fibrosis. The *adventitia* over the affected media was in all cases thickened, fibrotic and infiltrated focally with lymphocytes and usually also plasma cells. This was very marked in case 1, in which there were also a few minute foci of necrosis. The *intima* was always greatly thickened. This thickening was

essentially a hypertrophy, with secondary changes due to inflammation and degeneration. In early stages the intimal hypertrophy had an unusual appearance as shown in the ascending aorta in case 2 and the left external carotid artery in case 3. It contained numerous muscle fibres in an abundant mucoid matrix and the fibres were not arranged in circular and longitudinal layers. Collagen fibres were very scanty and delicate and elastic fibres were absent. Similar intimal hypertrophy is frequently seen in inflamed arteries in various sites such as gastric ulcer or the gall bladder, and it may occur in arteries showing chronic medial degeneration when this is accompanied by necrosis of groups of muscle cells in the media. Hypertrophy of this type is probably rapidly formed. The more common type of intimal hypertrophy, when not degenerated, shows an outer layer of longitudinal muscle and elastic fibres and an inner circular layer, the two being frequently separated by a prominent layer of elastic, the elastic stripe. Hypertrophy of this type is probably formed gradually. The appearance of the hypertrophied intima in my cases appeared to alter soon after hypertrophy had occurred. In the outer part of the intima, elastic fibres appeared and fibrosis replaced the muscle cells. This fibrosis might in part have been due to degeneration, since fibrosis is a common degeneration in hypertrophied intimas, but it was probably chiefly due to inflammation. Such appearances were present in the descending thoracic and abdominal aorta in case 2 and in the thickened segments of the internal carotid arteries in cases 2 and 3. At a later stage the intima became densely fibrotic throughout, as shown in the aorta in cases 1 and 4. Muscle fibres were greatly reduced in number and limited to the innermost part of the intima in case 1, but in case 4 they were still present in moderate numbers in all parts of the intima despite the marked fibrosis.

The *giant cells* which have been described were found only in the media, except in the internal carotid arteries in case 1, in which a few were also present in the intima. They were present in most but not all of the actively inflamed arteries. A few were of Langhans type; others were of foreign body type but of relatively small size. A few contained minute fragments of elastic fibres or were in contact with elastic fibres. It is doubtful, however, whether they were essentially connected with destruction of elastic tissue.

The most important effects of the arteritis were due to thrombosis of stenosed vessels. This was seen in cases 2 and 3, in which cerebral infarction occurred. Blindness due to blockage of vessels proximal to the central retinal arteries has also occurred. Definite aneurysm formation occurred in case 1 only, and rupture of one aneurysm led to fatal hæmorrhage. In case 4 dilatation of the abdominal aorta had been observed at laparotomy, but no aneurysm

was found at necropsy. It is possible that a moderate diffuse dilatation could occur during the active stage of the inflammation and disappear during healing, but not a saccular aneurysm. Since the inflammation tends to heal, one would expect to find occasionally at necropsy evidence of a previous arteritis in the aorta and elsewhere. Probably the effects of old aortitis of the type in question have been seen but were regarded as arteriosclerotic or syphilitic: If the Wassermann reaction was negative, excessive atheroma, loss of elasticity, extensive fibrotic intimal thickening or aneurysm formation in the aorta would make one suspect that there had been giant-cell chronic arteritis.

I regard the hyperpiesis present in case 1 as probably an effect of the arteritis due to implication of the carotid sinuses. The subject (aged 23) was very young to have hyperpiesis in the absence of renal disease, and this makes one suspect that it was of different ætiology from the hyperpiesia common in the middle-aged or old. The amyloidosis in case 4 was probably also secondary to the arteritis, since there was no other apparent cause and its distribution differed from that in so-called primary amyloidosis.

As regards ætiology little can be said. Syphilis had been excluded in three of my cases and in most of those in the literature. Tuberculosis may also be excluded. Neither tubercle bacilli nor other organisms could be found in sections in my cases or in those in the literature. In case 1 there was slight active tuberculosis in the cervical glands, and in cases 2, 3 and 4 and in Barnard's case healed tuberculosis was shown by calcareous nodules in the lungs and lymphatic glands, but the active and healed tuberculosis probably had no relation to the arteritis. The essential histological changes are those of inflammation and the prodromal symptoms commonly present strongly suggest an infection. Organisms of different kinds have been isolated from resected segments of temporal arteries but were probably contaminants. Whether or not the disease has a different ætiology from the other known types of arteritis must remain in doubt as long as there are types without known causation. The histological and clinical features of the disease, however, do not resemble those in other types of arteritis, and it is probable that the disease is a separate entity.

SUMMARY

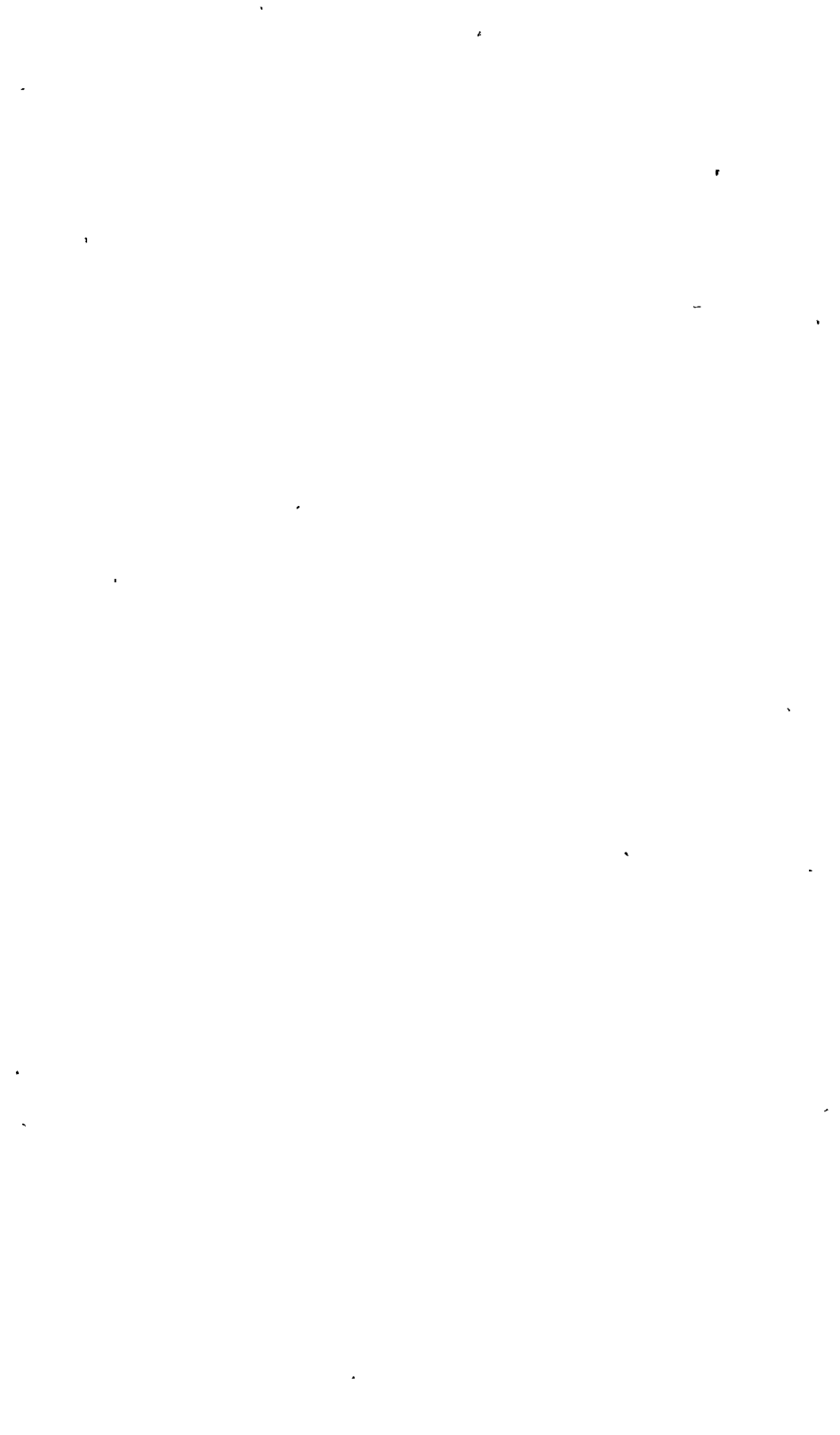
Four cases are described and a few others have been found in the literature of a little-known type of chronic arteritis. It affects the aorta, the branches of its arch and their branches, and possibly rarely other arteries. In the branches of the arch and in the internal and external carotid arteries its distribution is focal, bilateral and symmetrical, while in the aorta it is usually widespread. The

inflammation, given time, apparently heals, but fatal results have occurred from rupture of an aneurysm or from thrombosis during the stage of active inflammation. The ætiology is unknown. Because of the presence of multinuclear giant cells in most of the affected vessels, I have called the disease giant cell chronic arteritis.

I wish to thank Professor H. M. Turnbull for permission to publish cases 2 and 3 from the records of the Bernhard Baron Institute, the London Hospital, and Dr D. Romney for clinical details of case 1.

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SHORT ARTICLES

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THE ROLE OF IMPURITIES AND MIXTURES OF ISOMERS IN THE STAINING OF FAT BY COMMERCIAL SUDANS

W W KAY and RAYMOND WHITEHEAD

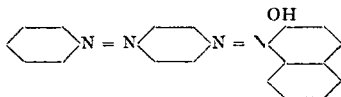
From the Department of Pathology, Victoria University of Manchester

In previous papers on fat stains we have studied sudan III (Kay and Whitehead, 1934) and given a technique for the use of sudan IV (Kay and Whitehead, 1935, 1937), in the present paper we show that commercial sudans give better fat staining than purified sudans owing to strengthening of the colour of stained fat by impurities

The chemical constitution of sudan III and sudan IV

Although the value of sudans as fat stains is enhanced by impurities, the strength of the colour imparted to fat depends also on the number of pure sudan dyes present, since mixtures of these dyes stain more strongly than the individual dyes alone. Sudan III is a single substance, sudan IV has various isomeric forms, some of which are likely to be found together in commercial products. It will be useful at this point to indicate the chemical constitution of the various pure sudans.

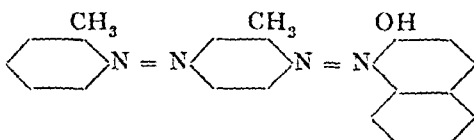
Sudan III is benzene <1 azo 1> benzene <4 azo 1> naphthol (2). It is prepared from amino azobenzene and β naphthol and is represented by the formula



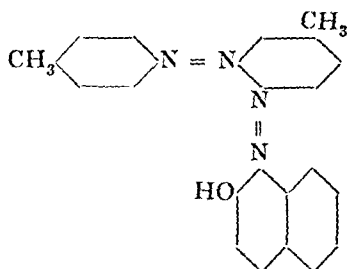
Its melting point is given as 195° C (Nietzki, 1880, Beilstein, 1933). The samples we prepared by an adaptation of Ruggli and Courtin's (1932) method for sudan IV isomer (1), after crystallisation from benzene, had melting points between 199.3° and 206.3° C (table I) and two commercial samples after purification melted at 205.1 and 206.1° C.

Sudan IV is prepared from amino azotoluene and β naphthol. Amino azotoluene is ordinarily prepared from *o* and *p* toluidines, theoretically, therefore, sudan IV may exist in four isomeric forms.

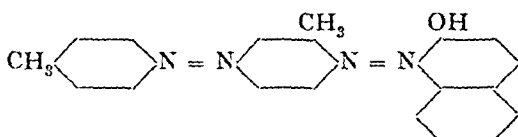
- (1) toluene - <2 azo 3> - toluene - <6 azo 1> - naphthol - (2), represented by the formula



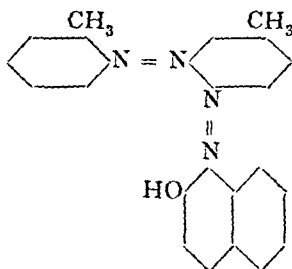
- (2) toluene - <4 azo 3> - toluene - <4 azo 1> - naphthol - (2), represented by the formula



- (3) toluene - <4 azo 3> - toluene - <6 azo 1> - naphthol - (2), represented by the formula



- (4) toluene - <2 azo 3> - toluene - <4 azo 1> - naphthol - (2), represented by the formula



Isomers (3) and (4) are extremely difficult to prepare; it is unlikely, therefore, that they are formed in the ordinary processes of manufacture. Isomers (1) and (2) are relatively easy to prepare and are therefore likely to be the dyes present in commercial sudan IV.

Isomer (1) is 2,2'-di-methyl-sudan III. Zincke and Lawson (1887) gave its melting point as 186° C., Ruggli and Courtin as 165-167° C., but Professor

Ruggli asks us to state that this figure should have been given as 186° C. Our product, prepared by the method of Ruggli and Courtin and crystallised from benzene, melted at 188.9–189.4° C.

Isomer (2) was prepared by the method of Zincke and Lawson who gave its melting point as 177° C., a figure confirmed by Ruggli and Courtin. Our product, crystallised from benzene, melted at 177.1–178.1° C.

Material and methods

The types of dye tested were (a) commercial products (b) purified dyes obtained by crystallisation from benzene or alcohol of commercial products or products made by us in the laboratory (c) the benzene or alcohol soluble residues after crystallisation of the purified dyes and (d) mixtures of purified sudans. Rabbit adrenals formed the test material. The technique of staining was that given in 1937 for our routine sudan IV. The stock solutions of (a), (b) and (c) were made by adding 2 g. of dye to 1 litre of absolute alcohol. The stock solutions of (d) were made by adding each ingredient in this proportion, thus in making a stock solution of two samples of sudan the total weight of dye added to a litre of alcohol would be 4 g. The stained sections were compared on a transilluminated sheet of opal glass and were also examined microscopically.

Melting points of the various dyes and nature of the colours they imparted to fat

Sudan III. Table I specifies the sudan III dyes tested and the colours they imparted to fat. It shows that of the eight purified dyes (B, D, H, I, K, L, M, N) all but three (H, M, N) melted at 204.7–206.3° C. On the general

TABLE I

Sudan III dyes and their fat staining properties

Reference letter	Description of dye	Melting point (°C.)
A	Sudan III, an English brand	
B	A crystallised from benzene	205.6–206.1
C	Benzene soluble residue after making B	
D	A different batch of A	
E	D crystallised from benzene	205.1–205.6
F	Benzene soluble residue after making E	
G	Sudan III, a German brand	
H	G crystallised from benzene	187.3–189.3
I	Sudan III, made by us from amino azobenzene purified by us, the sudan III being crystallised from benzene	205.3–206.3
J	Benzene soluble residue after crystallising I	163.5–164.5
K	I dissolved in H ₂ SO ₄ and precipitated by being poured into H ₂ O (regeneration—Hay and Whitehead 1934)	204.7–205.7
L	Repetition of I	204.7–205.2
M	Sudan III made by us from a sample of amino azobenzene different from that used to make I, the sudan III being crystallised from benzene	199.3–201.8
N	Sudan III made by us from amino azobenzene which we had prepared from aniline and purified, the sudan III being crystallised from benzene	199.3–201.8

Fat was stained red-orange by G, red by H and orange by all the others.

principle that a higher melting point indicates greater purity, our figures show that the accepted melting point of sudan III—195° C.—is at least 9° too low and was determined on dyes less pure than ours. Of the three purified dyes with lower melting points, H was entirely anomalous, melting 12° lower than any of the other dyes mentioned, and 17° lower than the purest of them. The melting points of the residues after purifying the dyes would be expected to be much lower than those of the purified dyes; J, for example, melted 42° lower than I, the corresponding purified dye.

Table I also shows that all the dyes except G and H stained fat orange, G staining it red-orange and H (G purified) red. The melting point and colour given by H make it almost indistinguishable from highly purified sudan IV isomer (1) (table II, T).

Sudan IV. Table II specifies the sudan IV dyes tested and the colours they imparted to fat. It shows that purification of our routine sudan IV (O) gave a dye (P) with a melting point intermediate between those of the

TABLE II
Sudan IV dyes and their fat-staining properties

Reference letter	Description of dye	Melting point (°C.)
O	Sudan IV, B.D.H. no. 555722, our routine fat dye
P	O crystallised from benzene	184.1-184.7
Q	Benzene-soluble residue after making P
R	A different batch of O crystallised from alcohol by The British Drug Houses Ltd.	161.6-165.6
S	Alcohol-soluble residue obtained by The British Drug Houses Ltd. after making R	...
T	Sudan IV isomer (1) made by us and purified by crystallisation from benzene	188.9-189.4
U	Sudan IV isomer (2) made by us and purified by crystallisation from benzene	177.1-178.1
V	Benzene-soluble residue after crystallising U . . .	160.2-164.2

Fat was stained red-orange by U and V, red by all the others.

sudan IV isomers T and U. The purified dye R obtained from another batch of the same brand of sudan IV melted 21° lower than P and was therefore less pure. The residue V melted 15° lower than the corresponding purified dye (U).

Table II also shows that all except two of the dyes stained fat red; the exceptions, highly purified sudan IV isomer (2) (U) and the corresponding residue (V), stained it red-orange.

Mixtures of purified sudans. Since, owing to its method of manufacture, commercial sudan IV is likely to contain more than one isomer, it was of interest to determine the fat-staining properties of mixtures of purified dyes. Of the four possible mixtures of I, T and U, IT and IU stained fat red-orange, TU and ITU red or red-orange according to the stock solution used.

Relative strengths of the colours given by the various types of dye

The relative strengths of the colours imparted to fat by the various types of dye were the same for both sudan III and sudan IV. Of the dyes listed in tables I and II, all except L, M, N and T belong to groups of either two or three genetically related dyes; A, for example, was the mother-substance of B and C. Three of the groups (A, B, C; D, E, F; O, P, Q) each

consist of commercial product, purified dye and residue, comparisons within each group showed that the purified dye stained less strongly than the commercial product and thus less strongly than the residue. The remaining groups consist of purified dye and residue only (I and K, J, R S, U, V) or commercial product and purified dye only (G, H), in each of these also the purified dye stained less strongly than the residue or commercial product. Of two purified dyes obtained from different batches of sudan IV, the dye shown by its melting point to be the purer (P) stained less strongly than the less pure (R). The strength of the colour imparted to fat is thus increased by the presence of impurities.

Each of the mixtures of purified sudans stained more strongly than any of its ingredients separately, the magnitude of the contribution of pure sudan to the strength of the colour given by commercial sudans may thus depend on the number of pure sudans in the dye.

Preferable properties of routine fat stains

As routine fat stains, (i) dyes staining red are preferable to those staining red orange or orange because red contrasts more sharply with the background of a section (ii) dyes staining strongly are preferable to those staining less strongly, and (iii) dyes giving moderately bright colours are preferable to those giving dull or murky colours. Of the various dyes tested, the only one possessing all these preferable properties was our routine sudan IV.

Discussion

The results show that commercial and laboratory preparations of sudan III and sudan IV can be separated by crystallisation from benzene or alcohol into two fractions one consisting mainly of pure sudan, the other (residue) containing most of the impurities. We do not know what percentage of pure sudan was present in the commercial products or residues or even in the purified dyes but to judge from the melting points our purified dyes seem to include the purest samples of sudan III and sudan IV so far described.

The impurities are by products of undetermined nature due to side reactions occurring during the process of manufacture, whether on the commercial or laboratory scale. The nature and relative amount of the impurities in the end product will depend on the nature and purity of the intermediates from which the dye is made and on the technique of manufacture. Variations in these factors would explain the variability of commercial sudans in composition and fat staining properties.

The results show that impurities play an important part in strengthening the colour imparted to fat by commercial sudans. The results of staining with mixtures of purified sudans show that pure sudan probably contributes more to colour strength in our routine sudan IV which is likely to contain more than one sudan than in commercial sudan III which contains only one.

It seems likely that in mixtures of sudans each individual sudan dissolves independently of the others and that the total amount of dye available for staining is therefore determined by the number of sudans present. Our results suggest that mixtures of commercial sudan III and commercial sudan IV might give colours even stronger than those given by commercial sudan IV alone.

Summary

- 1 The fat staining properties of fourteen sudan III dyes and eight sudan IV dyes have been studied.
- 2 Commercial and laboratory preparations of sudan dyes were separated

by crystallisation from benzene or alcohol into two fractions, one consisting mainly of pure sudan, the other (residue) containing most of the impurities.

3. Highly purified sudan III melted at 205.3-206.3° C., *i.e.* some 10° higher than the previously accepted figure, and stained fat orange.

4. Of two highly purified isomers of sudan IV, one, toluene- <2 azo 3>; toluene- <6 azo 1>-naphthol-(2), melted at 189° C. and stained fat red; the other, toluene- <4 azo 3>-toluene- <4 azo 1>-naphthol-(2), melted at 177-178° C. and stained fat red-orange.

5. Purified sudans stained less strongly than commercial sudans and these less strongly than residues.

6. Mixtures of purified sudans stained more strongly than their ingredients separately.

7. Impurities play an important part in strengthening the colour imparted to fat by commercial sudans.

8. Of the various dyes tested, our routine fat dye, sudan IV British Drug Houses no. 555722, was the most satisfactory as a fat stain.

We are indebted to The British Drug Houses Limited for various research materials and to Professor S. L. Baker for criticism.

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TWO OVARIAN TERATOMATA, ONE CONTAINING CEREBELLAR THE OTHER CEREBRAL CORTEX

H. F. BETTINGER

Department of Pathology, Women's Hospital, Melbourne

(PLATE XIX)

In 1939 R. A. Willis of Melbourne reported in this *Journal* the occurrence of well defined cerebellar tissue in a teratoma of the ovary. He was able to find only one other reference to a case of the kind (Askanazy, 1907), but even if a number of similar observations have gone unreported, the condition must certainly be one of great rarity. It is remarkable that a similar case should have come under observation in another Melbourne Hospital shortly afterwards and that about the same date at the same Hospital examination of another teratomatous ovarian tumour should reveal the presence of

cerebral cortex. This is a less infrequent occurrence than the development of cerebellar cortex but it is sufficiently rare to warrant inclusion in the present report, the more so as the two cases have other features in common.

Case 1

Clinical history A woman 26 years of age regularly attended the ante-natal clinic of this hospital during her first pregnancy. On examination a slight general contraction of the pelvis was found and a cystic tumour in the pouch of Douglas probably arising from the right ovary was detected. At term, the cyst prevented the head of the foetus from entering the pelvis and Caesarean section became necessary. A living male baby weighing 7 lb 2 oz was delivered and an ovarian cyst of about the size of an orange was removed. Recovery was uneventful.

Macroscopic description The specimen measured $10 \times 6 \times 6$ cm. On bisection it was found to consist of two cysts, the smaller, about 2 cm in diameter, being embedded in the wall of the larger. The main cyst had a thin smooth wall and was filled with clear colourless fluid. The smaller cyst, thicker walled, was stuffed with hair and sebaceous material.

Microscopic description The outer layers of the main cyst wall consist of strands of connective tissue in which lie islets of surviving ovarian tissue. The inner layers consist of nervous tissue of neuroglial type, nerve cells being absent. Occasionally some kind of ependymal lining can be seen but only in patches, not in the whole circumference of the cyst. No other tissues can be detected except in the wall dividing the two cysts. The smaller cyst is lined partly by well differentiated squamous epithelium partly by the granulation tissue with embedded hairs and foreign body giant cells, which so often lines these cysts. Beneath the epithelial lining is a layer of connective tissue containing hair follicles and sebaceous and sudoriferous glands. The most interesting part however is the dividing membrane between the two cysts. When approached from the smaller cyst this is seen to be composed firstly of a lining of well differentiated squamous epithelium, followed by a layer of connective tissue containing mainly hair follicles and sebaceous glands. Sudoriferous glands adjoin to form a rather wide layer. Groups of these glands are surrounded by smooth muscle and a large island of cartilage is present here. There follows a wide layer of adipose tissue and rather loose connective tissue which, somewhat deeper in the section differentiates into leptomeninges. These cover a layer of very well differentiated cerebellar cortex, of which the three strata, molecular, gangliosum and granular, are sharply demarcated. The whole arrangement reiterating most closely the architecture of the normal cerebellar cortex (figs 1 and 2). As the lumen of the main cyst is approached the cerebellar cortex loses its differentiation and blends with the ill defined neuroglial lining of this cyst.

Case 2

Clinical history Nurse P., 29 years of age, had noticed swelling of the abdomen for several months. No disturbance of general health was experienced, nor was there any menstrual irregularity. At operation a large cystic tumour of the right ovary was removed.

Macroscopic description The tumour was a rather thick walled cyst measuring some $24 \times 20 \times 8$ cm. On bisection a large amount of clear, slightly yellowish fluid escaped. Areas of calcification could be felt in the wall. At one place a cyst measuring $5 \times 4 \times 3$ cm was embedded in the wall of the main cyst. It was filled with sebaceous material and hairs. Close to it a tumour measuring $8 \times 7 \times 5$ cm projected into the interior of the main

cyst. Its cut surface showed some cysts but was composed mainly of solid whitish tissue.

Microscopic description. The wall of the small cyst has one structure which one would expect, namely skin and its appendages. The wall of the main cyst consists largely of dense fibrous tissue. There is no lining by skin; often the connective tissue itself forms the lining membrane. Here and there strips of neuroglial tissue similar to those in case I can be seen. The solid tumour within the cyst consists largely of nervous tissue. A glial felt-work, accumulations of nerve cells with brain sand between, and less differentiated nervous structures are the most frequent findings. Fair amounts of connective tissue and some adipose and muscular tissue are also present. In one very small area respiratory epithelium forms a canal surrounded by smooth muscle and mucous glands. The main feature of interest, however, is that quite frequently the nervous tissue takes the form of definite cerebral cortex (fig. 3). From loose connective tissue, leptomeninges have differentiated and cover structures closely imitating the architecture of the cerebral cortex. In its deeper layers this well defined tissue blends into less well defined structures, as in case I. It is noteworthy that the nearest approach to organoid structure again occurs in the vicinity of the dermoid cyst.

Comment

As a vivid imagination has led many authors to "recognise" all kinds of tissues and organs, even more or less complete parts of a foetus, in teratomata, rather rigid criteria have to be applied when dealing with structures hitherto rarely observed and insufficiently described. Of Askanazy's case neither a detailed description nor figures are available. In Willis's case, however, there is no doubt that the tissue described is cerebellar cortex. The gross architecture of the cerebellum, however, had not developed to a great degree, although there is some resemblance to an arbor vitæ. In this respect the present case I leads a step further, for not only the cytological but also the gross architecture of the cerebellar cortex is fully developed. Cystic teratomata which contain very little skin are somewhat rare, and cases like the one under discussion, where the main cyst is lined by nervous tissue, are not frequently seen. To what an extent such a development might proceed is illustrated by the second case. Here again the main cyst is not lined by skin. As in the first case, a small skin-lined cyst is included in the wall of the main cyst and although the latter is not completely lined by nervous tissue it can, from the lack of other structures, be safely assumed to be of neuro-epithelial origin. It is an unusual feature that the solid tumour inside the cyst consists to such an extent of brain tissue. While the formation of cerebral cortex itself is not an extraordinary rarity, the degree of differentiation reached in this case is seldom seen.

Summary

In the first of the two cases of ovarian teratoma here reported, the main interest lies in the development of unmistakable cerebellar cortex—the third recorded example. In the second case, interest centres in the preponderance of mature nervous tissue (including cerebral cortex) over all other tissue elements.

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BRAIN TISSUE IN TERATOMATA



FIG. 1—Above, squamous epithelial lining of smaller cyst with related epidermal structures below, teratomatous cerebellum



FIG. 3—Teratomatous cerebral cortex with leptomeninges



FIG. 2—Details of structure of teratomatous cerebellum showing Purkinje cells

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CORRELATION BETWEEN CARCINOGENIC POTENCY AND THE
FIRST SKIN REACTION TO CERTAIN HYDROCARBONS

B D PULLINGER

From the Imperial Cancer Research Fund, London

(PLATE XX)

A characteristic and possibly specific reaction to a single application of some carcinogenic hydrocarbons has previously been described (Pullinger, 1940). Only the most rapidly acting of these chemicals gave rise to this response and the dose required varied considerably. Experiments have now been done to determine the least dose of some of the rapidly acting hydrocarbons which will cause this early characteristic hyperplasia. The effect of excessive doses has also been observed.

A close correspondence was found to exist between the effective dose and the rate of tumour production by these compounds. The shorter the average latent period for carcinogenesis the lower was the dose required to cause the early characteristic response. There is a less close relationship to the percentage of tumour bearing animals. These comparisons are based on figures compiled by Iball (1939) and on reports of the activity of some recently synthesised compounds (Badger *et al.*, 1940).

All the compounds tested except benzpyrene and methylcholanthrene were supplied by Professors Cook and Kennaway. The data relating to carcinogenic potency are summarised in table I.

TABLE I

Compound	Latent period (days)	Percentage of tumours
5 9 10 Trimethyl 1 2 benzanthrane	31	65.0
9 10 Dimethyl 1 2 benzanthrane	43	65.0
Methylcholanthrene	109	88.5
Benzpyrene	109	78.0
3 4 5 6 Dibenzcarbazole	143	47.5
6 9 10 Trimethyl 1 2 benzanthrane	*	55.0
2 Methyl 3 4 benzphenanthrene	155	75.0
5 6 9 10 Tetramethyl 1 2 benzanthrane *	*	45.0

* Figures are not at present available owing to removal of the records to a safer place.

The findings relating to dose and response are shown in table II.

It has also been noticed that an excessive dose, about 6 times that required to produce a characteristic hyperplasia, sometimes but not always causes a different reaction. Instead of being hyperplastic the epithelium is reduced to one or two long thin flattened cells. These are stretched out over the sub-epithelial layers so that each covers a much larger surface than usual. A large amount of keratin lies over them but there are no signs of differentiation.

in the living cells and they are often so flattened as to appear atrophic (plate XX). The change is not general over the whole of the treated area; it occurs erratically here and there and it has not been possible to trace the stages through which it comes about. From the presence of excess of keratin and the absence of necrotic debris it is fairly certain that the larger dose is not on the whole a lethal one. Only a few nuclei undergoing karyolysis can be found. As in former experiments the impression is gained that these hydrocarbons are specific irritants which even in larger doses do not cause cell death.

TABLE II

Compound	Strength and amount of solution in acetone						
	0.025 per cent.	0.05 per cent.	0.1 per cent.				
	1 drop	1 drop	1 drop	2 drops	3 drops	6 drops	12 drops
5 : 9 : 10-Trimethyl-1 : 2-benzanthracene	-	+					
9 : 10-Dimethyl-1 : 2-benzanthracene		-	+				
Methylcholanthrene			+				
Benzpyrene				+			
3 : 4 : 5 : 6-Dibenzcarbazole					+		
6 : 9 : 10-Trimethyl-1 : 2-benzanthracene							+
2-Methyl-3 : 4-benzphenanthrene							+
5 : 6 : 9 : 10-Tetramethyl-1 : 2-benzanthracene							+

A plus sign indicates that the majority of the animals tested gave the reaction, a minus sign that none of them did so.

1 : 2-Azonaphthalene has been tested by the same technique. Even after an application of 12 consecutive drops it did not cause the hyperplasia characteristic of the most active carcinogenic compounds.

Summary

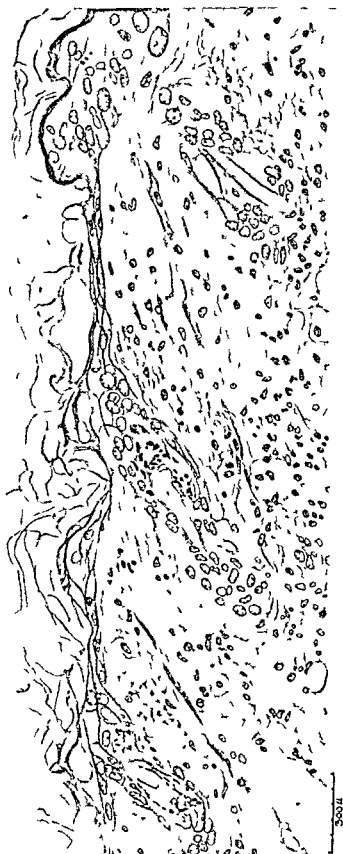
1. The shorter the latent period of a carcinogenic hydrocarbon, the smaller is the dose required to provoke the specific hyperplasia previously described.

2. Excessive doses of the same hydrocarbons are followed, in scattered areas, by thinning and stretching of epithelial cells so that they appear to be atrophic.

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 MARTIN, R. H., ROBINSON,
 A. M.

SKIN REACTION TO HYDROCARBONS



Mouo skin three days after the application of 18 drops of a 0.1 per cent solution of methylanthrene in acetone. Instead of a widespread uniform hyperplasia there are scattered patches of thin, stretched epithelium and of hyperplasia.



576 . 809 . 3

AN APPARATUS FOR THE "BREAKING DOWN" OF VACCINES
IN LARGE QUANTITIES

L S ASHCROFT and A H LEFMAIR

From the Bacteriological Department, Royal Naval Medical School

The apparatus described in this communication has been designed for the "breaking down" of any vaccine or combination of vaccines in large quantities in such a way as to reduce to a minimum the risks of external contamination and the amount of changing over from one bottle to another, as well as to effect economy in time and labour. Any number of bottles can be filled at one and the same time with one or more types of vaccine, and to any required total volume by using the necessary number of service feeds for connection to the required number of reception bottles. The various component parts are easily sterilised and can be quickly assembled and the risk of any external contamination after assembly is practically nil.

The diagram on p. 290 shows the apparatus completely fitted up for filling 4 bottles, each of 4000 c.c. capacity, with mixed and diluted T A B C vaccine from vessels containing strong separate stock suspensions of *Bact. typhosum* and *Bact. paratyphosum* A, B and C.

A is a 200 c.c. burette, graduated in 2 c.c. divisions with a two way glass stopcock (a), and is plugged at the top with sterilised cotton wool, the whole being fixed on a metal tripod. The burette is connected by rubber tubing (b) to a 5 way glass Carrel distributing tube, from which one rubber tube leads to B, a 4 litre bottle containing saline, and the remaining four to the bottles containing the stock suspensions. Each stock bottle, as well as bottle B, is further connected by rubber tubing to a second 5 way Carrel tube (c) leading to a filter bottle (C) which is connected to a blower.

The burette (A) is connected by tube (d) to a 4 way Carrel tube (e) which leads by separate tubing to the four 4000 c.c. bottles 1, 2, 3 and 4, for the final reception of the combined and diluted T A B C vaccine.

We have found that a Stuart pump with a small Klaxon motor is a satisfactory blower, the one we use has worked satisfactorily and continuously for the past 18 months, giving a steady and even flow of liquid. It is essential that fine bore rubber tubing (3.5 mm.) should be used. Metal screw clamps are fitted on all tubing leading from the stock bottles, the saline bottle and the Carrel tube (e). These are very much more satisfactory than ordinary spring clips, which are not easily worked and are liable to slip. They need not be sterilised.

The practical working of the apparatus

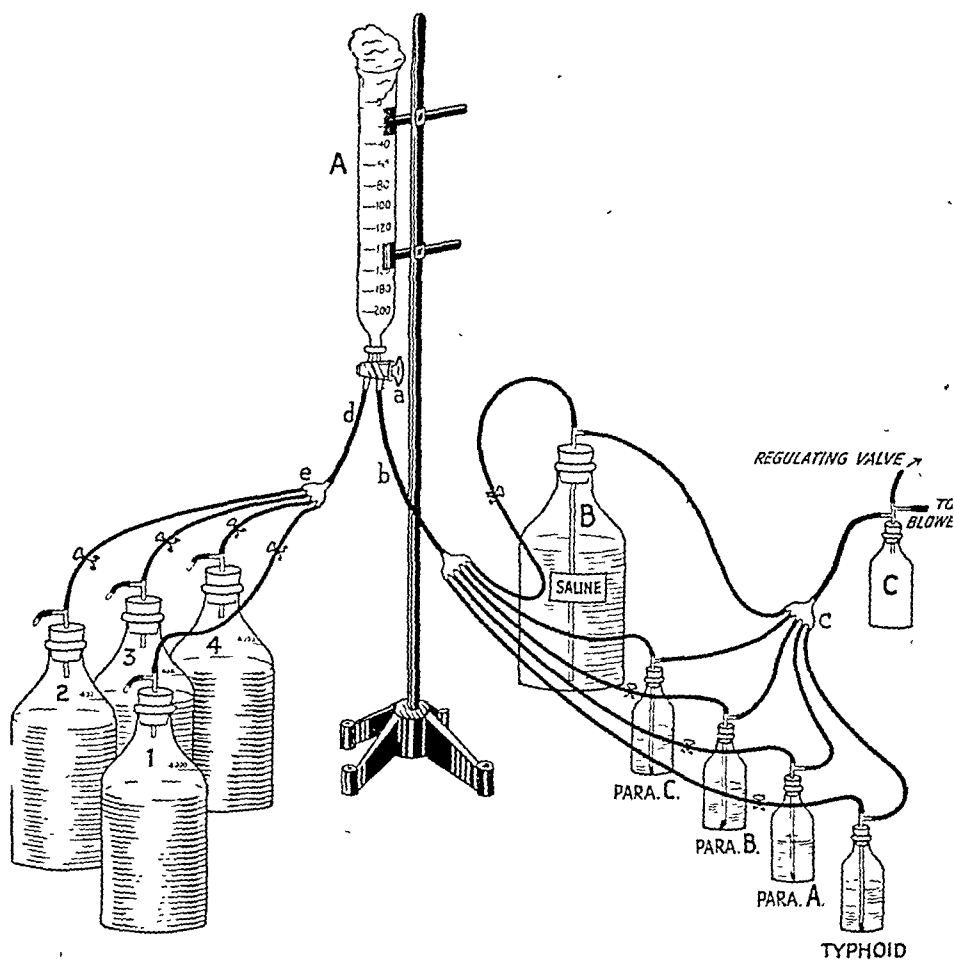
I. Open the clamp on bottle B and pump about 20 c.c. of saline into burette A. Close a. Remove the cotton wool plug and add the required amount of phenol to 1, replace the plug.

II. Open the clamp leading to bottle 1, connect A with tube d and allow the contents of A to flow into bottle 1. Close clamp and stopcock. Repeat procedures I and II for bottles 2, 3 and 4.

III. With the clamp on bottle B closed, open the clamp on the typhoid bottle and pump into it sufficient vaccine for the four reception bottles, say 168 c.c. (42 c.c. for each bottle). Close the connections between A and the typhoid bottle and, by opening the appropriate taps and clips, allow

42 c.c. of suspension to run from *A* to each of bottles 1-4. Repeat the same process for each of the suspensions paratyphoid A, B, and C.

IV. All four reception bottles having received the required amounts of each suspension, all that remains to do is to pump through the burette, into each reception bottle, enough saline to fill it up to the 4000 c.c. mark.



A, burette; *B*, saline; *C*, filter bottle; *a*, stopcock; *b* and *d*, connections leading from burette; *c* and *e*, Carrel tubes; 1, 2, 3 and 4, reception bottles; typhoid and para A, B and C, bottles containing stock suspensions.

By using this apparatus, 16,000 c.c. of vaccine can be "broken down" in a little over half-an-hour by three people: with two people working, the time taken is a little longer.

Each bottle is fitted with a metal cap of appropriate size with a standard fitting, transmitting a two-way tube. The length of the rubber tubing on the various pieces of the apparatus is roughly 12-15 inches.

For sterilisation, the rubber tubing, Carrel tubes, bottle fittings and burette are all assembled as in the diagram. Each metal cap is separately wrapped in Kraft paper, and the ends of the rubber tubing attached to the

smaller metal tops for the stock bottles are inserted into test-tubes which are also separately wrapped in Kraft paper, so that each piece of the apparatus can be undone separately. The whole apparatus is sterilised in the autoclave at 15 lb pressure for 45 minutes.

576 . 851 . 4 (*Bact. typhi*) : 576 . 809 . 75

THE ACTION OF SALICYLATES ON THE DEVELOPMENT OF ANTIBODIES FOLLOWING ANTI-TYPHOID INOCULATION

C BRUCE PERRY

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Despite the specific action of salicylates in relieving the pain and fever of acute rheumatism very little is known of their mode of action. It has been suggested (Poynton and Schlesinger, 1937) that the relapse of acute rheumatism which so frequently occurs in a rheumatic child after an acute streptococcal infection may be prevented by the administration of salicylates for some weeks after the streptococcal illness. Evidence has been put forward (Schlesinger and Signy, 1933, Perry, 1939) that this prophylactic action is associated with the inhibition of antibody formation. Swift (1922) appears to have been the first to study the action of salicylates on the formation of immune bodies. From a considerable number of experiments of various types he concluded that the administration of salicylates materially reduced the amount of antibody produced in response to many antigens. Derick, Hitchcock and Swift (1927-28) extended these observations to man. They found that large doses of aspirin given to patients who had received horse serum largely prevented the development of the arthritis of serum sickness, the other manifestations being unaffected, and that, associated with this phenomenon, the development of anti-horse precipitin in the serum of treated patients was very much reduced or absent.

It appeared desirable to obtain further information upon this action of salicylates and a group of medical students who were receiving anti-typhoid inoculation volunteered to assist. There were 16 in all, 6 men and 10 women. Eight (3 men and 5 women) agreed to take aspirin continuously for the 10 days between the first and second inoculations. Four (3 men and 1 woman) took 45 grains daily and the remainder (4 women) only 30 grains daily. The serum was tested before the first injection and as far as possible twice weekly for the first two weeks and then at less regular intervals. The examination after the second inoculation was somewhat interrupted by rather heavy rains and the Christmas vacation. The sera were tested against H suspensions of *Bact. typhosum* and *Bact. paratyphosum* B obtained from the standards laboratory at Oxford. Tests were also made against *Bact. typhosum* O suspensions, but in many of the cases the development of O agglutinins was missed owing to their late appearance and short duration. Before the first injection none of the sera showed any agglutinins, with the exception of that of one woman who had received anti-typhoid inoculation 12 years previously. In this student there was agglutination of the *Bact. paratyphosum* B suspension at a dilution of 1:10.

In the control cases receiving no aspirin, agglutinins for *Bact. typhosum* H first appeared 4 days after the injection and rose rapidly to their maximum titre by the 8th to the 11th day. The maximum titres observed varied in different individuals from 1:500 to 1:10,000. Observations after the second injection were not so detailed but no higher titres were found. The

titre gradually fell and 10 weeks after the first injection varied from 1:250 to 1:1000. The response to *Bact. paratyphosum* B was almost identical except that the maximum titres varied from 1:500 to 1:2500; at the end of 10 weeks the titres ranged from 1:100 to 1:500. In the group receiving aspirin the time of development of agglutinins was almost identical, as was also the titre reached, the maximum for *Bact. typhosum* H varying from 1:500 to 1:10,000 and the 10 weeks titre from 1:50 to 1:1000. The maximum titres for *Bact. paratyphosum* B were 1:500-1:5000 and the 10 weeks titres 1:25-1:250. The chart shows the distribution of the different titres observed in both groups of subjects. This shows that there is no difference in the initial response or maximum titres reached but does suggest that the treated cases fell to a rather lower titre in 8-10 weeks than

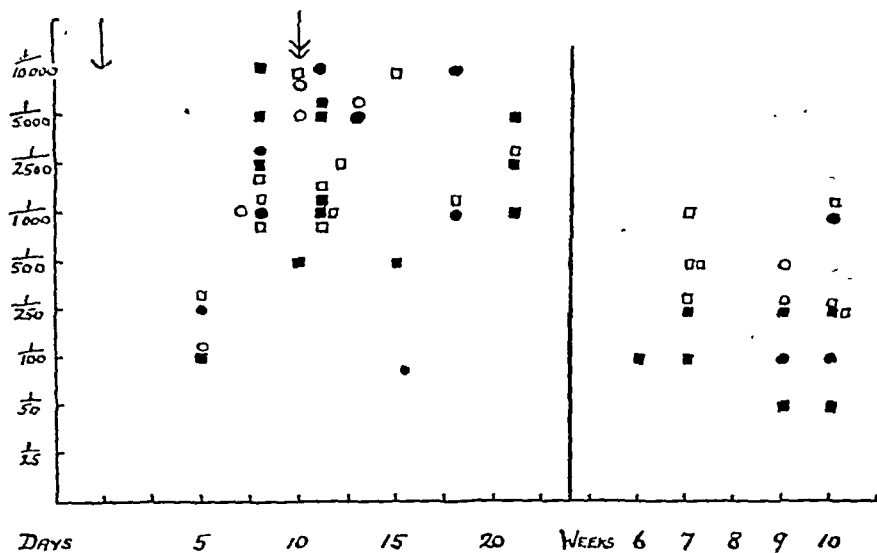


Chart showing the distribution of the various titres of typhoid H agglutinins observed in control and treated subjects.

□ = Female control

○ = Male control

■ = Female receiving aspirin

● = Male receiving aspirin

↓ = First inoculation

↓ = Second inoculation

the controls. This difference is, however, too small to have any statistical significance.

There was thus no evidence that the administration of aspirin in the doses given had any appreciable effect on the development of H antibodies in response to anti-typhoid inoculation. The dose of aspirin was small compared with that used by Derick *et al.* but not dissimilar to that used by Schlesinger and Signy, and it was as large as was considered desirable in ambulant persons at work. Whether the different results are due to the smaller dose of aspirin, the different antigen, or the large dose of antigen used is not clear. Swift's original experiments with various antigens were performed with very small numbers of animals and it is doubtful if they would stand statistical analysis. On the other hand the results of the observations of Derick *et al.* on anti-horse precipitins appear to be conclusive, though whether salicylates in sufficient dosage are capable of inhibiting antibody formation in all circumstances must remain undecided.

Conclusion

The administration of aspirin in doses of 30-45 grams per day had no appreciable effect on the development of agglutinins following typhoid inoculation.

Thanks are due to the students who so kindly made the investigation possible and to Dr K. E. Cooper for his constant interest and advice.

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576 809.34

SOME USES OF NIGROSIN IN BACTERIOLOGY

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In the period before 1914 it was possible to obtain commercially India ink which, when spread in a thin film on a microscope slide, gave a reasonably homogeneous background. After 1919 this was not in my experience possible and accordingly I looked around for other substances which gave homogeneous dark backgrounds and tried, among other things, nigrosin. This is an almost black dye which, when spread on a film and dried, makes a perfectly homogeneous film and does not stain bacteria.

For these reasons nigrosin is very suitable, as a substitute for India ink, for providing a dark background for unstained bacteria. It has the advantage that it furnishes a smooth background and can readily be removed by water, so that a bacterial film after examination in nigrosin, can be washed free of this substance and stained by any of the usual methods.

For over twenty years I have been teaching the use of negative staining by nigrosin in the preliminary examination of a culture and for other purposes, and the object of this article is to encourage a wider use of the method which, after long experience, I have found most valuable.

Preparation of the nigrosin solution

The following solution is prepared

Nigrosin, water soluble (Gurr)	10 g
Water	90 c.c.
Formalin	10 "

The formalin is added merely as a preservative to prevent the growth of moulds and bacteria.

While a 10 per cent solution of nigrosin is most suitable for general

purposes there are occasions when a saturated solution is preferable, and at other times the 10 per cent. solution can with advantage be diluted with distilled water.

Demonstration of the size, shape and arrangement of bacteria

This can be done in two ways.

(a) A suspension of bacteria in water is spread on a slide as if for staining and allowed to dry. A small drop of nigrosin is then placed on one side of the bacterial film and is spread over the bacteria with a loop or another slide. This film can be dried by gentle heat over the bunsen flame and is then ready for examination.

(b) A small drop of the nigrosin solution is placed on a slide and into this are mixed bacteria from the culture (just as if it were a drop of water). When the mixture is complete the drop can be treated in either of two ways. (i) With another slide a film is made in the same way as a blood film. This gives the most perfect results if the technician is skilled but otherwise may be disappointing, the film being too thick or too thin. (ii) The nigrosin-bacteria mixture is spread out on the slide by a circular motion of the loop, which gradually advances along the slide from its original position until it no longer contains sufficient fluid to leave a film. This gives an uneven film—the first portion is too thick, the final portion too thin, but somewhere in the middle there is a perfect thickness. By this method several different colonies can easily be examined on the same slide.

The thickness of the nigrosin film is important. If it is too thick the bacteria are partially overlaid by the nigrosin so that they appear too small. Suppose, for instance, we take a film of staphylococcus in nigrosin. The staphylococcus is about $0.8\ \mu$ in diameter. If the nigrosin dries in a film of the same thickness the staphylococcus can only appear as a very minute clear spot with a hazy outline round. If the film of nigrosin is too thin the background may not be perceptible, but in this direction there is an enormous margin. Even if the amount is so small that it dries into a hardly perceptible film the bacteria can still be seen, as around each bacterium the depth of fluid approximates by capillary attraction to the depth of the bacterium. When this dries the bacterium is surrounded by an easily perceptible dark halo inside which is a clear area denoting the size and shape of the organism.

It is customary in the examination of cultures, especially by students, to use for a preliminary examination a film stained with a simple stain such as methylene blue. The method described above has distinct advantages over simple staining. The whole operation takes less time, and is wholly performed on the worker's bench, thus avoiding the necessity of walking to a sink to stain the specimen. The outlines of the bacteria are more clearly defined than by any staining method. Degenerate bacteria which have lost their staining properties still show up as faint shadows in the nigrosin film. Sometimes it is difficult, with stained specimens of tiny colonies, to focus accurately on the portion of the slide on which the film is spread. If the film is spread in nigrosin solution the black colour makes it easy to find.

Use of negative staining with nigrosin in combination with positive staining methods

After a bacterial film has been stained with Gram or other stain it is sometimes an advantage to spread nigrosin over half of the stained film. This leaves half of the film for examination as a stained film while in the other half there is the combination of positive and negative staining. Nigrosin is, however, a decolourising agent and rapidly removes most stains from

bacteria, so that if the stain is to be preserved the nigrosin film must be rapidly dried. Even then partial decolourisation frequently occurs. This partial decolourisation in some cases gives very beautiful results in demonstrating granules in, for example, a Gram-stained film of the diphtheria bacillus.

This method involves so little extra labour that it is worth doing when stained films of material like faecal suspensions or direct smears of diphtheria swabs are being examined. It has the advantage of showing up organisms like spirochaetes which do not show up prominently in the stained film.

The demonstration of spores

The usual method for the demonstration of spores is to stain with hot carbol-fuchsin, decolourise with weak acid, and counterstain the bodies of the bacilli with a stain such as methylene blue. If we consider the structure of an anthrax bacillus we see that if the spore is stained with fuchsin and the bacillary body with methylene blue the edges of the spore must be rendered to some extent indefinite by being overlaid by the blue coloured body of the bacillus. Theoretically, therefore, the spore would be more clearly shown if the body of the bacillus were left uncoloured and its outlines delineated by negative staining with nigrosin. This theoretical consideration is borne out in practice.

The details of the methods of staining recommended are as follows.

For single slides. A bacterial film is made in the usual way on a slide and dried. Boiling carbol-fuchsin * (or carbol-fuchsin diluted with an equal amount of water) is poured on the slide and with occasional warming is left for 5 minutes. The stain is washed off with water and the film rapidly washed with alcohol, which removes the deposit of stain adhering to the slide.

Decolourisation can be effected by covering the slide with 10 per cent. nigrosin solution for 5 minutes or longer, after which it is washed off with tap water and the film is dried. A drop of nigrosin (10 per cent.) is then placed on one end of the slide, and with another slide is spread as a film over the stained specimen and allowed to dry. This gives bright red spores and unstained bacterial bodies, the outlines of which are clearly seen against the nigrosin background.

If it is desired to decolourise more rapidly, the usual reagents such as weak acid or sodium sulphite may be used. The decolourising agent is washed off with water, the slide is dried, and then nigrosin solution is spread over the film and allowed to dry. The primary decolourisation need not be quite complete, as in the few seconds which the nigrosin takes to dry it completes the removal of the stain from the bacillary bodies.

When the stronger decolourising agents are used some of the stain may be removed from the spores, these do not then show the intense bright red colour which is seen when nigrosin is the only decolourising agent used.

For large numbers of slides for class purposes. From a culture on a suitable solid medium the bacteria are transferred to water in a test-tube to form a thick suspension. To this is added an equal volume of carbol-fuchsin. The whole is boiled for about 5 minutes or immersed in a saucepan of boiling water for anything over 5 minutes. The tube is now filled up with water and centrifuged, when the bacteria rapidly sediment, as they are agglomerated in masses. The excess of stain is removed by washing once or twice with water, which process, owing to the agglomeration of the bacteria, can usually

* Basic fuchsin	1 g.
Phenol (pure)	5 g.
Alcohol	10 c.c.
Water (distilled)	100 c.c.

be done without centrifuging. Almost all the fluid is now removed, and with a pipette or glass rod the bacterial masses are, as far as possible, broken up into a suspension. Approximately 2 volumes of 10 per cent. nigrosin solution are now added and mixed with the suspension. After about ten minutes (the time varies considerably with the type of organism) decolourisation is complete, and small drops are placed on slides and spread into films of such a thickness that the nigrosin furnishes a suitably thick background. The films are allowed to dry and are ready for examination.

Bacteria stained in this way show clear cut, intensely red spores in uncoloured bacterial bodies sharply defined in the nigrosin. The picture is a more definite one than when a double stain is used, and the edge of the spore is very sharp, making it obvious that it is a definite body and not merely an indefinite red area in a blue bacillus. This makes it easier to convince students that spores are real entities, and the method also makes it possible to prepare without undue trouble a sufficient number of stained specimens to present one to each student, so that there is no excuse for their not having seen spores.

The demonstration of capsules

Negative staining for the demonstration of capsules is a common procedure. The bacteria are usually mixed with a drop of India ink and spread in a film on a slide which is then stained. Nigrosin cannot be used in this way as, being water-soluble, it washes off the slide in the process of staining. It can be used, however, in another way, by spreading it in a film over the stained slide of capsulated organisms, when it settles down and dries in a film outside the capsule, thus leaving an unstained area between the nigrosin and the stained bacterial body which represents the capsule.

The decolourising effect of nigrosin, so useful in the demonstration of spores, is here a disadvantage as, unless great care is exercised, the stain may be removed wholly or partially from the bacteria. The stains which I have found to be most resistant to the decolourising action are Gram's stain and methylene blue.

A film of the bacteria is made on a slide in the usual way, stained by Gram or methylene blue and dried. Then a small drop of nigrosin is placed on the slide and with the edge of a second slide is drawn into a thin film over the upper half of the stained specimen. This should be dried rapidly by heat to minimise the risk of decolourisation by the nigrosin.

If the edge of the nigrosin-covered portion of the film is examined with an oil immersion objective one half of the film shows stained bacteria and negatively stained capsules, while the other half is merely a stained specimen of the bacterial bodies. This demonstration of the capsules in half of the field is very convincing to the sceptical student.

This method, while very beautiful, is a dangerous one for general use until we have a stain which is not removed by nigrosin, as partial decolourisation may, unless careful observation is made, closely simulate capsulation. It is, however, free from the objection which may sometimes be levelled against the method of staining the bacteria in India ink films, namely that the bacteria have shrunk away from the ink in the process of drying, as the nigrosin is only applied to the film after the bacteria are dry.

Summary

Nigrosin solution is recommended for the negative staining of bacteria. It may be used by itself as the simplest and most effective method of demonstrating the size, shape and arrangement of bacteria, or it may be combined with staining methods to demonstrate spores or capsules.

OBITUARY NOTICES OF DECEASED MEMBERS

William Bulloch

1868-1941

(PLATE XXI)

WILLIAM BULLOCH, emeritus professor of bacteriology in the University of London died on 11th February at the age of 72. Bulloch was appointed bacteriologist to the London Hospital on 27th June 1897. He became Goldsmith's professor of bacteriology (London University) in the London Hospital Medical College in 1919, and retired on 30th September 1934, becoming consultant bacteriologist to the Hospital and emeritus professor. He continued to work in his old laboratory until war broke out and finally returned to his Hospital for a small operation and died. He was thus actively associated with one institution for 42 years.

Bulloch was born at Aberdeen on 19th August 1868 the younger son of the late John Bulloch and brother of the late John Malcolm Bulloch the historian of the Gordons. He studied medicine at Aberdeen qualifying in 1890 with highest honours. He was awarded the Murray medal as the most distinguished graduate of the year. He took his M.D. in 1894 also with highest honours, and won the Struthers gold medal in anatomy.

After qualification Bulloch worked for nine months with D. J. Hamilton in the pathological department at Aberdeen and for some short period as an assistant in private practice. According to his own confession this latter exploit was not a success and in 1892 he went on a speculative trip to Germany and became a voluntary assistant to Birch-Hirschfeld in Leipzig. Here he published a paper with Schmorl. In 1893 he studied pathological anatomy in Vienna and at the same time attended lectures on general medicine. Returning to Aberdeen he came to London with Arthur Keith in 1894, and worked with David Ferrier at King's College on neuropathology. In the same year he was appointed assistant to Victor Horsley, the professor of pathology in University College, but before starting his duties he took the course of bacteriology at the Pasteur Institute given by Roux and Metchnikoff. He taught pathology and bacteriology at University College during the winter session 1894-95.

His work with Victor Horsley on neuropathology was not congenial to him and in July 1895 he was appointed assistant

bacteriologist at the antitoxin laboratories of the British Institute of Preventive Medicine (now the Lister Institute), where, after the retirement of Ruffer, the director, in 1896, he assumed entire charge of the serum department. In the next year he started his long association with the London Hospital as bacteriologist to the Hospital and lecturer on bacteriology and pathological chemistry at the College, an association which was to last his working life.

In an attempt to assess the effect of Bulloch upon the movements of his time, mention should be made of his activities as director, as teacher, as research worker, as administrator, as bibliographer and as historian.

As director

Bulloch was one of the first to be appointed as a salaried specialist in contact with patients at a London teaching hospital. Although in the medical school he took a natural place alongside the teachers of anatomy and physiology, Arthur Keith and Leonard Hill, in the hospital he found himself an exponent of a new subject which was already taught in a rudimentary and no doubt mangled way by such younger members of the honorary staff as were interested. It must be confessed that Bulloch had a passionate regard for accuracy in detail which induced him even in later life to point out errors with devastating precision, but in later life he did so with greater authority and more humour than he could command in his earlier days. Those most intimate with him knew well that his early impatience was due to private misfortune, but those who did not, resented it. Thus Bulloch became involved in animosities with various members of the honorary staff, though certainly not with all, which detracted from his ability to take his place in the hospital as a director of pathology in general. He withdrew into the fastness of his own department and posted a notice on his door to deter the adventurous who overcame his last steep flight of stairs.

It is perhaps doubtful whether Bulloch at any time aspired to be the director of a large department. One tends to picture him as a student of the printed word—a bibliophile—impatient of interruption by outside events. One doubts whether, even if his sunny temperament had not suffered a temporary eclipse, he would have been torn gladly from his books by the exactions of executive work. However, Bulloch did not become director of a large department. He started with three rooms in 1897 and accepted two others in the early nineteen-hundreds for the accommodation of opsonic work; otherwise his department displayed none of those expansions which are now commonplaces in pathological circles. Certainly plans were made and drawn out on more than one

occasion in an attempt to include him in the natural evolution of pathology in a hospital, but he would not rise, and he remained sitting for nearly 40 years in the same chair in the draught of an open door, in the worst of his five rooms, becoming happier and happier, but not advancing the interests of his department. Routine executive matters and routine work, the selection of staff, ordering of goods, determination of methods, he left entirely to his assistants.

Bulloch in no way directed research in the sense of making suggestions in a positive way for lines of work or for the solution of difficulties. He was purely critical and sometimes depressing, but if a paper survived without comment one could at least be tolerably satisfied that no one else could pick it to pieces. He had, up to the last war, such a respect for the printed word, especially German, that any manuscript which purported to contain a new discovery or to run counter to published views was apt to be regarded with suspicion. He would suggest from his prodigious memory and knowledge of the literature that your new discovery was to be found in Baumgarten vol —, p —. Hurried and anxious investigation showed that the volume and page were correct but that the subject matter did not of course deal with the precise point you were making. As the years went on and the older literature became outmoded, he seldom commented on the work done in his laboratory, and one might think that he had not quite gathered what it was about had not one heard from other sources that Bulloch had been boasting about the wonders we had produced.

Thus Bulloch as a director did not belong to that dynamic class which subdues its assistants by intellectual eminence. Neither was he a nonentity. He was a person sitting apart from the daily difficulties, who had to be satisfied before we could be satisfied. We had not only a respect for his opinions, tempered by a recognition of his foibles, but we had a respect for his honesty. And, finally, he had the important gift of keeping us happy.

As teacher

Throughout his life Bulloch personally delivered all lectures on bacteriology and general pathology at the Medical School and also supervised the practical classes. He would not have been human if, in his later years, his lectures had exhibited a complete recasting, but though they remained of the same structure he brought them up to date along orthodox lines. Certain passages always remained, always had the same effect on his audience, and always revealed to us in the room next door that he was lecturing, for instance, on the gonococcus. His audiences were always attentive, there was always a look in his eye of something to come. I have only known

one student so somnolent that he resorted to a pointed glass rod to reach him across the table. It is not always the case with lecturers on medicine that they have any particular relation to the matters they propound, but with Bulloch one immediately recognised that here was a man who knew what he was talking about, who talked about it without fear or favour, who, by means of deft historical touches, appeared so much a part of his subject that one wondered why he did not come in with Pasteur and Koch.

Though he had a strong sense of the dramatic and produced his effects well, he was not an orator. His language, difficult at first for an Englishman to follow, was simple and natural but endowed curiously with an arresting quality which held the attention. He was equally successful in his public lectures—for instance, in his Horace Dobell lectures at the Royal College of Physicians in 1910, his Tyndall lectures at the Royal Institution in 1922, and recently (1936) in his Heath Clark lectures. The fact is that he enjoyed lecturing and was a good lecturer.

As research worker

Bulloch's research work consisted largely of papers on pathological anatomy, of which probably the most outstanding was his paper with Sequeira in 1905 on the relation of the adrenals to the sexual organs. On the bacteriological side he published nothing of great importance himself, and only a few papers with such colleagues as Twort, Craw, Western and Atkin. His career as an original worker covered only the first ten years; afterwards he applied himself to critical compilations and reviews. Bulloch had at his disposal ample time for engaging in any work he preferred, but in fact he had no desire for personal research, at any rate at the bench.

It may be imagined that a mind so critical and so versed in the works of others might hesitate to commit himself to the hazards which fringe this path. The necessary culmination of research work is the printed account, from which there is no retreat, and Bulloch himself would hardly admit that the author of a printed howler could ever regain the respect of his fellows. Holding the views that he did, a mistake would be disastrous.

In what may be called research involving the literature, the weighing of printed evidence and the drawing of guarded conclusions within the limits of the printed evidence, he had an abiding interest. After his first ten years he more or less devoted himself to this aspect of his work. He wrote notable articles in Allbutt & Rolleston's *System of Medicine*, and a number of contributions to Karl Pearson's *Treasury of Human Inheritance*. In these latter, however, he was clearly more interested in the bibliographical aspects

of the work than in the pathological. He contributed no personal observations, and at any rate in one case left the whole of the general account and conclusions to his junior collaborator.

As administrator

Bulloch served for many years and very assiduously on a number of important committees and consultative bodies. He attended regularly as consultant at the King Edward Sanatorium at Midhurst. He was a member of the Executive Committee of the Imperial Cancer Research Fund, a member of the Advisory Board of the Best Memorial Fellowships, resigning last year after 29 years' service, a member of the Government Committee on Foot and Mouth Disease, an original member of the Medical Research Committee and of their Bacteriology Committee, president of the pathological section of the Royal Society of Medicine. In 1918 he became a member of the Lister Institute and in 1932 chairman of the Governing Body, which office he retained to the end.

Bulloch took his duties on these bodies seriously, but was never disposed to take himself too seriously. His wide knowledge was always available and lucidly expounded, but he did not readily take part in discussions, especially in later years when perhaps he felt that things were getting a bit beyond him. He had quite a flair, when it became a question of allotting grants to individuals, for summing up quite forcibly the merits or demerits of a candidate. It was sometimes not obvious on what he based his conclusions, but events usually confirmed his views, and in any case he was always manifestly honest. Actually, his power to sum up a character was a reflection of one of his chief interests in life. He had a great liking for the study of the innumerable types he came across, and this seemed to be one of the chief reasons which induced him to attend so many committees. One used to see him sitting at the table quietly observing with his beady eye the foibles of his colleagues rather than attending to the matter in hand, and often enough after the meeting he would take them off in his inimitable way with such sympathy as to suggest that he envied them their characteristics. He seldom met anyone for the first time without contriving to know something about them, and he earned their appreciation by firing at them a series of intelligent questions and getting to know more. He had an amazing memory. He never forgot anyone he had met once, and he could reproduce incidents in the past life of old colleagues which they themselves had forgotten or remembered only sufficiently well to be able to recognise that truth was not entirely submerged in art. It is not out of place in an appreciation of his services to committees to point out here that he was excellent company, with a fund of stories which never

palled, and that this was one of the reasons why he was so valuable. He kept people human and kept them awake.

As bibliographer

Bulloch's bibliographical knowledge of medical literature, and not only that of his own subject, was remarkable. He had developed a technique based on the catalogue of the Surgeon General's Library in Washington and the old *Index Medicus*: this enabled him to get in touch with the key papers, which he then pursued in the Library of the College of Surgeons or the Patent Office and later in the Royal Society of Medicine. This was before the day when a librarian could be asked to attend to these matters and before the day of most of the present abstracting journals. He practically never consulted an abstract and deplored the tendency of others to do so. The idea of getting someone else to look up the literature of a subject was abhorrent to him. His method was personal study of original sources, and thus he avoided the transmission of mistakes. No act of a colleague was more likely to incur his displeasure or cause him to express it, than a bibliographical error. In fact he often checked up the "literature" at the end of a paper before he read the body of it, to judge whether the author was reliable or not. He thought, and with reason, that anyone who could be guilty of being slipshod in such matters was likely to be slipshod in other things. In later years the increasing size of the library and the bibliographical amenities of the Royal Society of Medicine attracted Bulloch more than the older libraries, and for ten years from 1925 he acted as honorary librarian.

Bulloch's greatest bibliographical feats, which are unlikely to be surpassed, appeared in his article on hæmophilia in the *Treasury of Human Inheritance* and in the Medical Research Council's "Diphtheria". Some of his colleagues, finding him immersed in the private lives of obscure hæmophilics, could not quite appreciate his object in struggling through such a mass of stuff. His object was to achieve finality in a matter which had often been attempted before—an accurate description of the data contained in every single published paper. He got within a half dozen. In "Diphtheria" he contributed 100 pages of bibliography to 426 pages of text by seven authors. All papers, except thirteen, were consulted "with care" in the original, but, it will be understood, not consulted to obtain data useful in the deliberations of his co-authors, but consulted to ensure bibliographical accuracy.

As historian

It is probably as a historian that Bulloch's name will be most remembered after his personality has faded away. But though his

last work — *The History of Bacteriology* — gives an admirable summary of the earlier developmental trends in this new subject, his chief delight was the recording of the lives and characters of individuals. His *History* contains some 50 pages of biographical notices of bacteriologists, mostly deceased, and even though these are very short they represent the result of considerable labour. These notices are, however, mere "lives" without the "characters". They are the bare bones without the flesh with which we know well he could have clothed them. It would appear that the private lives of bacteriologists are, or at least were bordering on the scandalous, and thus much of the fruits of Bulloch's gleeful researches have not been published. It is understood that he left behind a mass of data, and it may be hoped that some of this, after expurgation, may appear in print.

Apart from his *History*, which was founded on the historical notes which he contributed to the *System of Bacteriology* of the Medical Research Council, Bulloch contributed to the journals no less than twelve appreciations or obituary notices of bacteriological "characters" and colleagues, a task for which he was specially suitable as so many of them were personally known to him.

He was judged worthy of the Fellowship of the Royal Society (1913) and of the LL.D. of his Alma Mater (1920) while our own Society, of which he was an original member, elected him an honorary member in 1938.

In the writer's view Bulloch will not be remembered by posterity for any achievement in pathology but for his contributions to history, and without any doubt that was his aspiration. His contemporaries are, however, able to take into account attributes of which posterity cannot judge. Neither his reputation as a historian or bibliographer, nor his reputation as a pathologist, accounts for the respect in which we held him. With us Bulloch's chief asset was his personality. He was a man of impressive learning who nevertheless behaved with simplicity and even levity without losing the respect of his juniors. He had many friends in all classes and in all spheres. He was approachable by all and cultivated all. If he did not influence all, he influenced many.

In 1923 Bulloch married Irene Adelaide, widow of Mr A. A. Baker, and entered upon a period of serenity which even the slow progress of his last illness did not affect.

We offer our sympathy to Mrs Bulloch and to her daughter, Mrs Clifford Dobell.

P. T.

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As Stanley Griffith

Arthur Stanley Griffith

1875-1941

(PLATE XXII)

By the death of Stanley Griffith, pathology has suffered a sad loss. Ever since 1903, when he began to work for the Royal Commission on Tuberculosis, he had devoted himself almost entirely to the study of the various kinds of tubercle bacilli and other acid fast micro organisms and their relation to the different manifestations of tuberculous disease in man and animals. Although owing to his retiring disposition, he was known intimately to but few of his colleagues, he had acquired an international reputation as the leading authority on his subject, and he had made friendly contact with fellow workers in Paris, Berlin and Copenhagen. It is to him mainly that we owe our knowledge of the proportion of deaths caused by bovine infection in each of the kinds of tuberculosis occurring in man. Thus he found varied in different places, being considerably higher in Scotland and some of the northern parts of England than elsewhere. His most important discovery together with W. T. Munro, was that a not inconsiderable number of cases of chronic pulmonary tuberculosis were caused by the bovine type of tubercle bacillus. This was contrary to the opinion previously held, that practically all such cases were derived from other similar cases. Among the 2371 cases of the kind from Scotland examined by himself and his colleagues, the proportion infected with the bovine bacillus was found to be as high as 5.4 per cent.

Though some of us may not agree with his opinion that the bovine type of tubercle bacillus is at least as virulent for man as the human type, if not more so, we must all admit that Griffith has shown that infection of man from the tuberculous cow is far from insignificant, and that Koch for once, was wrong when he declared at the British Congress on Tuberculosis held in London in 1901, that the risk of infection of man by the milk and flesh of tuberculous cattle was hardly greater than the risk of hereditary transmission, and that he did not deem it necessary to take any measures against it. If Griffith had done nothing else, he has earned our gratitude by showing how necessary it is to guard the purity of our milk.

Griffith was the first to show that a large proportion (about one half) of cases of lupus were due to the bovine type of the tubercle bacillus. This, I think, was quite unexpected. He was also interested in attenuated strains of tubercle bacilli of both human and bovine type, the majority of which have come from cases of lupus. He also studied the value of immunisation with the

attenuated strain of Calmette and Guérin, as well as the more recently discovered vole bacillus of Wells, which he considered more efficient as an immunising agent than the former. He also showed the danger of attempting to immunise cattle with the human type of bacillus, and pointed out that it might get into the milk of the heifer and even affect its calf.

Griffith died at the age of 66. Up to August 1939 he had been full of work. He had gone to Saltdean, near Brighton, for the week-end, when he was seized with a heart attack which was followed by cerebral embolism. He made a partial recovery and returned to Cambridge in November. Though not paralysed, or at least only to a trifling extent, his speech was slightly affected. His condition improved from time to time, but at intervals there were set-backs, and from the first there was no hope of his complete recovery. For eight months or more he was confined to bed. Nevertheless he kept cheerful, his mind was as bright as ever and he was busy writing and correcting papers for the press right up to the day of his death. It was a mercy that he passed away peacefully on 9th April before the tragic death of his brother Fred, to whom he was devoted, and who was killed with Dr W. M. Scott in an air-raid on London a few days later.

Griffith was born at Ecclestone in Lancashire in 1875. He was educated locally and at the Liverpool Institute, Medical School and Royal Infirmary, Liverpool. He took his M.B. and Ch.B. with second-class honours in 1897 and his M.D. (Vict.) in 1901. He took the D.P.H. of the English Royal Colleges in 1902 and the Ph.D. of Cambridge in 1926. He held the Derby Exhibition in Medicine at Liverpool in 1897 and was resident house physician to the Royal Infirmary and medical officer to the Lock Hospital in Liverpool in 1898. From 1899 to 1902 he held the Alexander Fellowship in Pathology.

Through the influence of Professor Boyce he became, in 1903, one of the scientific investigators of the Royal Commission on Tuberculosis, which had been set up in consequence of Koch's announcement at the Tuberculosis Congress already mentioned. The Commission had two experimental farms at Stansted which had been placed at their disposal by Sir James, afterwards Lord, Blyth. Griffith went at first to Walpole Farm, where material from tuberculous animals was being investigated, but after a year he moved to Blythwood Farm, which dealt with material from cases of the human disease. For seven years he worked for the Commission, and when it finally made its report he came, at the invitation of Sir G. Sims Woodhead, to Cambridge. In the small corrugated-iron laboratory buildings which the Commission had built at Blythwood, and which had been removed to the Field Laboratories on the Milton Road about a mile or two from

Cambridge, Griffith worked for the rest of his life, first as Grocer's scholar from 1911 to 1914, and then as a member of the external scientific staff of the Medical Research Council, in whose service he remained until his death. Since 1920 he had been a member of their Tuberculosis Committee. In 1927 he was awarded the Weber-Parkes medal of the Royal College of Physicians for his work on tuberculosis and in 1939 was made a Companion of the Order of the British Empire. After coming to Cambridge he joined Magdalene College. In 1927 he married Mrs A. N. Beech.

Griffith was exceedingly careful and painstaking in all he did, and he devoted himself almost exclusively to his work. His outside interests were few, though he loved to roam on the Sussex Downs during his short and infrequent holidays, and he was an interested member of the Cambridge Antiquarian Society. I do not remember his ever playing golf, but he enjoyed lawn tennis in his younger days and he kept a canoe for journeys on the Granta. His home, Paradise House, Newnham, was on an island almost covered by trees. Latterly his only hobby was bee keeping, and there were many hives in his woodland garden. I sometimes think he was like his bees, immersed in his one peculiar task and caring little for anything but that and his home.

He is survived by Mrs Griffith and one son aged 12. Though known intimately by few, he was greatly loved by those who were privileged to come into contact with him, and by them he will be sorely missed. He was an original member of our Society.

L. C.

To many pathologists Arthur Stanley Griffith was no more than a name, a name most honourably associated with a lifetime devoted to the bacteriology of tuberculosis.

Even in Cambridge few of his colleagues or of his neighbours knew him really well, but to many people he was a familiar figure as he passed on his bicycle along the Backs every morning to his work, every evening to his home, with the punctual regularity by which all his life was ordered.

Precise, formal, almost cold, but always courteous in his dealings with his fellow men, he did nothing to invite friendship, but the few who surmounted the barrier of his reticence were rewarded by a loyalty as generous as it was undemonstrative. He was in fact a kindhearted but most modest man and, if his colleagues often found that he was slow, even unwilling, to undertake a suggested task, they knew that his reluctance was due to an unshared doubt of his own ability to fulfil their wish, for he would give nothing but his best and he judged himself by no low standard. A lecture to students, the reading of a paper to a society, even an informal talk on his own work cost him a great effort and hours and days of

preparation. But on the rare occasions when he was persuaded to talk about his work, he expressed in a few well weighed words the results of much and most careful work. His published papers, the record of the labours of a lifetime, have been and will remain a most important source of our knowledge of the bacteriology of tuberculosis.

H. R. D.

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William McDonald Scott

1884-1941

(PLATE XXIII)

OUR Society has sustained a great loss by the death on the night of 16th-17th April, through enemy action, of Dr W. M. Scott. He has been described as one of the best all-round bacteriologists in this country, an opinion with which few of us will disagree. His knowledge in the field of bacteriology as applied to epidemiology and preventive medicine was unrivalled and during the last ten years he had come to be regarded both in this country and abroad as the authority on the *Salmonella* and dysentery groups of bacteria.

William McDonald Scott was born in Nyasaland in 1884 of Scots parents. He spent his early boyhood in Dumfriesshire, where his father had settled in general practice, and he was educated at Dumfries Academy and the University of Edinburgh, which he entered in 1900. After a brilliant academic career he graduated M.B., Ch.B. with first class honours in 1905. In the following year he took the Diploma of Tropical Medicine and Hygiene and in 1907 graduated B.Sc. (Public Health). From 1906 to 1908 as McCunn Research Scholar Scott worked, at first under Professor Hunter Stewart in Edinburgh and later under Professors Gruber and Emmerich in the Hygienisches Institut of the University of Munich, on the conditions affecting the persistence of typhoid bacilli in contaminated soil. Thus early he became interested in the practical application of bacteriology in relation to the spread of infectious disease. During part of his stay in Munich he also worked in the chemical laboratory of Professor Soxhlet on the quantitative transfer of decomposition products of protein by water currents in the soil. From 1908 to 1910 he worked as Carnegie Research Scholar in the research laboratory of the Royal College of Physicians of Edinburgh under Professor James Ritchie on the identification of bacterial species by serological methods, a subject on which he was later to become an acknowledged master. At this period he became interested in the mechanism of the anaphylactic reaction. His interpretation of careful and detailed experiments on the rabbit has been recognised by subsequent workers in this field as essentially correct and the work formed part of his thesis which was submitted for the M.D. degree of the University of Edinburgh in 1910, and which gained for him the award of a gold medal.

From Edinburgh Scott moved to Cambridge where, as John Lucas Walker Student in Pathology (1910-12), he worked under Sims Woodhead. His principal research during this period was on the effects of ultra-violet light on the physico-chemical properties



Wm. Scott

of proteins in solution, and he also carried out experiments on the mechanism of the disinfectant action of such rays on water—a procedure which in recent years has become one of the recognised methods of disinfecting the water of swimming baths. In 1911 he worked for some months with Professor Besredka in Metchnikoff's laboratory at the Pasteur Institute in Paris on the behaviour of trypanosomes in immunity reactions. In 1912 he went as Government Pathologist to the Straits Settlements and during the sixteen months that he held this post he made a special study of amœbic and bacillary dysentery, a disease the bacteriology of which was later to occupy much of his attention. From the temporary post in the Straits Settlements he returned to Cambridge as assistant to Sims Woodhead and carried out a research on the specific bactericidal properties of quinine derivatives.

In 1914 Scott became medical officer of health to the North-East Kent United Districts. While holding this office he was invited to collaborate with the bacteriologists in the Local Government Board laboratory in an investigation of the mode of spread of cerebrospinal fever and thus began his close association with Fred Griffith which endured for the rest of their lives. The conclusions reached in this enquiry were taken as the basis for administrative action in relation to this disease and for the application of serum therapy. In 1917 Scott resigned his post with the North-East Kent authority on his appointment to the permanent staff of the Local Government Board laboratory which became in 1919 the pathological laboratory of the Ministry of Health. In this Service he remained until his death. His earlier training and experience and his natural ability qualified him for the eminent position which he was to occupy in the Ministry of Health laboratory as an authority to whom appeal could be made in the wide field of bacteriology in its relation to public health.

Latterly much of his time was devoted to the organisation and administration of the Emergency Public Health Laboratory Service which came into being on the outbreak of war and just before his death he was appointed senior medical officer in charge of the newly created Laboratory Division of the Ministry of Health.

Although the list of Scott's publications is not a very long one and is by no means a complete measure of his services to British bacteriology, it serves to indicate the wide field which his work covered. He never lost his interest in the subject of anaphylaxis, which had engaged his attention in his earlier years; his contribution on "Anaphylaxis and related phenomena" in the Medical Research Council's *System of bacteriology* published in 1931 is a complete and critical account of the subject at that time and is regarded by many as one of the best sections in the *System*. The studies on meningococci made in collaboration with F. Griffith

between 1915 and 1917 involved a detailed examination by cultural and serological methods of many strains of meningococci isolated from the cerebrospinal fluid and from the nasopharynx. Using methods of agglutination and absorption of agglutinins, meningococci were divided into two main serological groups. Many of the strains isolated from the normal nasopharynx fell into the same serological groups as did those from the spinal fluid of cases of meningitis. On reading their reports it is difficult to escape the conclusion that little new has since been added to this subject.

Many of Scott's published papers deal with the identification of pathogenic bacteria by serological means, for he appreciated that only by exact knowledge along these lines could many of the problems in the epidemiology and control of infectious diseases be solved. His work on the serological characters of influenza and diphtheria bacilli had no obvious practical applications in the field of public health but similar studies on the *Salmonella* have been of great scientific and practical interest. He quickly appreciated the importance of Sir Frederick Andrewes's work on diphasic variation and applied the new knowledge to the identification of difficult *Salmonella* strains. In differentiating the Thomson type of *Salmonella* he introduced the technique of growing the organisms, which appeared to possess only group flagellar antigens, in a medium containing group anti-serum and by subsequent colony selection succeeded in obtaining cultures which possessed a hitherto unknown specific antigen. Scott became the acknowledged expert in this field of bacteriology, and in collaboration with others defined several new *Salmonella* types or determined the association of types previously found only in animals with human infections. In an excellent article contributed to the *British Medical Journal* in 1930 he drew attention to the association of eggs, especially duck eggs, with food poisoning in man and was later able to prove, through the isolation of *Bact. typhi murium* from duck eggs and from the ducks of three flocks concerned with outbreaks of food poisoning, that eggs were the vehicle of infection. Scott excelled in the diagnosis of *Salmonella* and dysentery organisms by slide agglutination of non-lactose-fermenting colonies direct from plates of McConkey's agar. In his search for a "specific" *Salmonella* colony in stool cultures from cases of food poisoning, he would examine with infinite patience upwards of a dozen colonies, testing each one with half-a-dozen different sera from the assorted collection of bottles kept in a wooden box always to be found on his bench. He had a flair for choosing the right sera, and time and again he was able to diagnose and report from direct cultures the presence of an "Aertrycke", a "Newport", a "Dublin" or a "Sonne" or "Flexner" within twenty-four hours of receiving the specimens.

During the last twenty years he played an important part in

the investigation of every outbreak of enteric fever and food poisoning in which the assistance of the Ministry of Health had been invoked. In the bacteriological examination of water supplies, he acted as an official and unofficial court of appeal in cases of doubt or difficulty, and in this capacity he helped in the preparation of the memorandum issued by the Ministry on "The bacteriological examination of water supplies", in which a standard method of bacteriological testing of water was proposed. The method has been widely adopted in this and other countries. He was a member of the Preventive Medicine Committee of the Medical Research Council and the Nomenclature Committee and Salmonella Sub-Committee of the International Association for Microbiology, and he served on the technical committee on scientific glassware of the British Standards Institution.

In research work Scott was accurate in his methods, resourceful in planning experiments and untiring in their prosecution, not one of his findings has been called in question. Although in later years he devoted much of his attention to the Salmonella and dysentery bacilli, those who knew him appreciated that he had a comprehensive knowledge of the whole field of bacteriology such as few of his contemporaries could claim. Like many others he was never fond of writing papers but his style was simple, lucid and forceful. His articles of a general character such as those in the *Lancet* on "Bacillary dysentery" and "The enteric fevers" are clear and effective. The discussion on the aetiology of influenza in the section on "The influenza group of bacteria" in the *System of bacteriology* is an excellent critical review of the evidence available on the subject before the later work on influenza viruses.

Scott's position at the Ministry of Health brought him into contact with medical officers of health and public health bacteriologists throughout the country and many have occasion to be grateful for his expert guidance and assistance. He seemed to know so many things not to be found in text books. His help was frequently sought in the investigation of animal diseases and his opinion was greatly valued by workers in this field. He was, perhaps, more closely in touch with both veterinary and medical bacteriologists than any other man in the country.

The team at Dudley House was a happy one and the dry wit and keen sense of humour of both Scott and Griffith infused an atmosphere of cheerfulness into the grim and almost sordid surroundings in which they worked. Visitors to the Ministry of Health laboratory, young or old, from this country or from abroad, will have pleasant recollections of their meetings with Scott. None visited the laboratory but went away with new knowledge or the wiser for his kindly counsel. Completely unselfish, he would go to great pains to help others and was without the most unassuming of men.

Scott was a man of generous nature and liked the company of his fellows. The same friendly atmosphere which one encountered at the laboratory was even more in evidence in Dulwich or in Oxford, where many friends enjoyed the cheerful hospitality of his home. His sly humour, frequently exercised at the expense of a member of his family, was appreciated alike by the victim and by other members of his household.

A good linguist, he spoke and wrote French almost like a native. He read widely and kept abreast of modern English and French literature. Apart from reading, his hobbies were gardening and bee-keeping, and much of his spare time was devoted to his large garden and orchard at Dulwich. He enjoyed a game of tennis with the younger members of his family, and could sing a Scottish song with gusto.

Scott's promotion to the post of senior medical officer in the Ministry of Health just before his death was a well merited reward for his great services to bacteriology and to public health in the Department which he had helped so much to build up. It was for him a matter for special gratification that the importance of the bacteriological laboratory in public health work was officially recognised by the creation of a Laboratory Division in the Ministry of Health and it brought nearer to realisation his unselfish ambition for a national laboratory service.

The gap left in our ranks by Scott's death will be very difficult to fill and to his many friends among pathologists, both medical and veterinary, his passing has left a very deep sense of personal loss.

When working at the Pasteur Institute in 1911 Scott met his future wife, Mlle. Mollard, the daughter of a secretary of the Senate. In addition to his widow he leaves four daughters and three sons; his eldest son, Major A. Scott, is at present serving abroad with the R.A.M.C.

V. D. A.

A. W. D.

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Francis Temple Grey

1886-1941

WHEN Francis Temple Grey (barrister-at-law, deputy coroner for East Middlesex, and pathologist to the Princess Elizabeth of York Hospital for Children and the East End Maternity Hospital) died suddenly and unexpectedly in January of this year at the early age of 54 the forensic and pathological branches of the medical profession lost an able and a learned man. His friends and colleagues are missing something more—a deeply loyal, remarkable and in some ways intriguing personality. To his colleagues of the children's hospital at Shadwell he was above all things a first class morbid anatomist and biochemist. His extensive knowledge was so widely related that he was always able to bring it into vivid relationship with clinical problems. He always seemed to gravitate to clinical work whenever the opportunity arose, and in talking to him one sometimes got the impression that he regarded himself as primarily a clinician driven by fate to work in the post-mortem room. It is not surprising then that the bulk of his forty or fifty original papers deal directly with biochemistry in relation to clinical problems.

Born in South Kensington, he was educated at St Paul's, Versailles and then went to Australia, where he studied medicine at Sydney University. At Sydney Hospital he was successively house surgeon, medical registrar and resident pathologist. After the last war he went to Oxford, and read law at Wadham College, where he took a first.

When this war started and the staff at Shadwell was depleted, he organised the out patient department and did, competently and accurately, a great deal of the work himself, and its present comparatively flourishing condition is in great part due to him. At the East End Maternity Hospital he was regarded not only as a pathologist, but, in an emergency, as a competent anaesthetist and obstetrician as well.

At the time of his death he was at work on the notes and records of over 5000 post mortems which he had hoped to incorporate in a book on morbid anatomy. His knowledge of histopathology and bacteriology was not nearly so extensive. This, however, did not limit his usefulness, for he never pretended to anybody, least of all to himself, that he knew when he did not.

In the forensic field, coroners and magistrates soon discovered that he was a witness of unshakable reliability and he gained an increasingly enviable reputation with both bench and bar. He had written for the 1929 edition of the *Encyclopædia Britannica* the articles on medical jurisprudence, capital punishment, coroners and lunacy law. Members of the Grotius Society knew him as an authority on the law of extradition and on legal problems relating to territorial waters and the air.

A remarkable linguist, he spoke French, Italian, German and Spanish fluently, Russian fairly well, and he could read Portuguese, Dutch and modern Greek. He never made a speech that was not well salted with quotations from the classics. He was an expert yachtsman and held a first mate's certificate. While a surgeon in the Royal Navy he was mentioned in dispatches for conspicuous devotion to duty when H.M.S. *Cornwallis* was sunk. In addition to all this, he and his wife were the centre of a large and spontaneously happy family who will miss him deeply and long.

One may well ask how it is that a person so able and accomplished was relatively unknown except in one or two rather small circles. The answer lies in the fact that he was interested in relating the data which came under his observation to established existing standards, and to do this he used all the resources of an able and widely stocked mind. But his mind was not speculative, it did not occur to him seriously to question the validity of the existing framework of society, he wanted to conserve and strengthen rather than to change and extend. This is well illustrated by his pride in the fact that he was an active inspector in the special constabulary. None who knew him would think that this pride was the result of the power it brought rather it represented, perhaps quite subconsciously, his own very humble picture of himself in relation to the society in which he lived. One other fact gives an interesting glimpse into a character simple and singularly free from modern conflicts. He was the possessor of an

old, beautifully hand-worked sextant. "It is" he once said to me "the apple of my eye—it has no index error".

His calm acceptance of things as they were was sometimes apt to irritate, but such irritation was merely passing and was quickly obliterated by the memory of much grave and courteous kindness, often almost unnoticed because it was a spontaneous and natural expression of his inner conception of his ordinary duties as a citizen. This is how he would best like to be remembered.

H. W. S. WRIGHT.

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SPUTUM FILM CULTURES OF TUBERCLE BACILLI: A METHOD FOR THE EARLY OBSERVATION OF GROWTH

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(PLATES XXIV-XXVII)

EVEN with the best methods of cultivation the growth of tubercle bacilli is slow. For this reason, if experimental and diagnostic results are to be obtained more quickly, it is necessary to use some method of micro-culture. The observer must provide himself with a microscope and a method which will enable him to recognise growth whilst it is still invisible to the naked eye.

Several methods of micro-culture have already been described. Wright (1924) and Fleming used slide cells and capillary pipettes filled with whole blood inoculated with tubercle bacilli. The blood was allowed to clot and the colonies which grew were stained with Ziehl-Neelsen after dissolving away the hæmoglobin. Hesse, Meissner and Quast (1928) modified this technique and made an extensive investigation of the effect of dyes. Kahn (1929), using Chambers's micro-manipulation apparatus, observed the growth of individual tubercle bacilli isolated in micro-droplets and described a life cycle.

It is very doubtful if growth is any more rapid with micro-methods of culture than on ordinary media such as Petraghani's or Loewenstein's. Growth at first follows a geometrical progression and can be represented as a logarithmic curve. At first individual tubercle bacilli are visible with the $\frac{1}{12}$ in. objective. After some weeks colonies become visible to the naked eye. But long before this, if an appropriate technique is employed, colonies may be rendered visible with a low power objective. The micro-methods

enable one to recognise growth more quickly—in a few days instead of weeks. It is merely a question of magnification.

None of the previous micro-methods, however, conveniently permit observation of tubercle bacilli in sputum or other pathological material which is infected not only with tubercle bacilli but also with other organisms. They are purely experimental methods, using an inoculum of tubercle bacilli from a culture previously obtained in the orthodox way. The present method involves a new principle of technique which enables one to work with sputum.

This new principle is very simple and is merely that tuberculous sputum films dried on glass may be treated with acid to destroy other organisms than the tubercle bacillus and incubated in contact with some suitable culture medium such as hæmolyised blood, the tubercle bacilli being thereby transformed in a matter of days into micro-colonies which can be stained with Ziehl-Neelsen and which are easily visible with the low powers of the microscope. Whilst most of the work has been done with sputum the method is also applicable to pus and caseous material. With material which is not secondarily infected preliminary treatment with acid is unnecessary.

Obvious signs of growth can be seen with the $\frac{1}{2}$ in. objective in 24 hours. Colonies may be just perceptible with the $\frac{2}{3}$ in. objective in 48 hours. In 3 days they are usually quite distinct. Sometimes the bacilli appear to wake up and start growing at different times so that in 3 days only a few colonies may be visible, and in 5 days there may be considerable disparity in size. Each day's incubation makes a great difference to the size of the colonies whilst growth is in its logarithmic phase. In a week the cultures are well grown. The typical appearance of cultures at this time is shown in figs. 1-3.

The tubercle bacilli do not appear to attack the mucus. They form colonies which merely lie embedded in it. Unstained early colonies appear as irregular clefts in the film. For this reason the bulk of the colonies in a week-old culture are in no way affected by the movements of the medium or the process of washing. The supernatant medium of luxuriant week-old cultures in Petri dishes can, however, be shown by centrifugalisation to contain bacillary masses derived from some of the colonies which happen to lie on the surface. After more than one week's incubation the large coiled-up colonies tend to rise above the upper surface of the film of mucus so that in the process of preparation of the stained film a scattering of free bacilli may be seen.

The acid, besides killing the non-acid-fast organisms, exerts a coagulating effect on mucus, which is particularly well marked with the stronger solutions. This is an advantage, as the process is one of "fixation". Most of the cellular elements of the sputum

FILM CULTURES OF TUBERCLE BACILLI

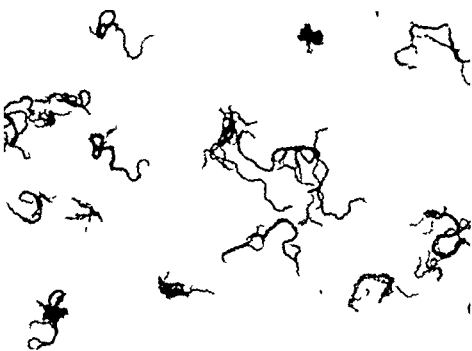


FIG 1

FIG. 2.

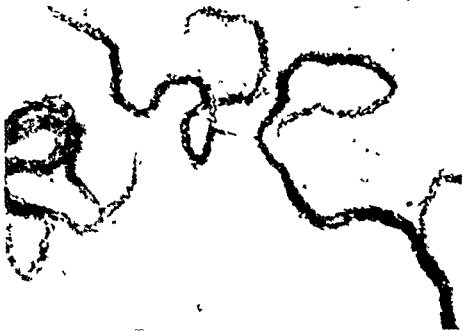
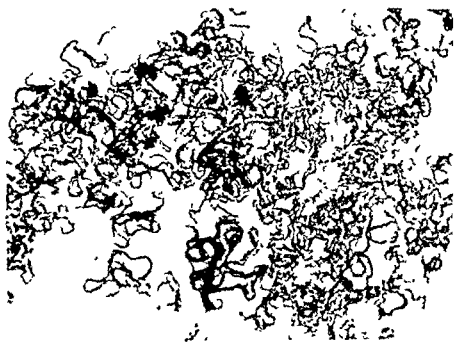


FIG 3

film autolyse (figs. 4-7). Pus cells disappear rapidly but epithelial squames persist longer and may still be visible after a week. Sputum which has been liquefied by the action of bacteria is difficult to work with, as the films disintegrate.

Much latitude is permissible in the use of acid. In one experiment with 3 sputa the same results were obtained with 5 per cent. sulphuric acid as with 25 per cent. Treatment with 30 per cent. sulphuric acid was definitely harmful, but some slight growth with two of the specimens was obtained even after this. On the only occasion when 35 per cent. sulphuric acid was used it caused the sputum films to break up.

The subsequent removal of the acid must be thoroughly carried out. With blood as medium, the acid left may attack the hæmoglobin immediately, but concentrations insufficient to do this can inhibit growth. Many early failures were due entirely to this cause. Neutralisation is inadvisable, as alkalis dissolve mucus. Reliance must be placed on thorough washing with distilled water. At least two changes of water are needed and sufficient time (several minutes) must be taken over the process for the acid to diffuse out of the film.

The only media which have been used so far are human blood and its derivatives. Whilst blood may not prove to be the best medium, it has in its favour that it is always to hand. Successful growth can be obtained with blood (whole, defibrinated or lysed) obtained by digital puncture, but it is perhaps better to get it by venepuncture. Stored citrated blood too old for therapeutic use serves admirably. Growth with serum alone is very poor. Citrated and defibrinated blood give equally good results, but the growth is best when the blood is lysed. Blood may be lysed with an equal volume of distilled water, but better lysis and possibly better growth is obtained with a similar amount of a 1 per cent. solution of saponin. The clarity of saponin-treated blood, however, is its chief advantage. Fleming, at a laboratory demonstration of the Pathological Section of the Royal Society of Medicine in 1937, showed that in a capillary tube tubercle bacilli grow much more rapidly in blood laked with saponin than in whole blood, or in blood in which the leucocytes had been killed by other means.

Although growth may occur in the presence of contamination, it is usually diminished or completely inhibited. Contamination may dissolve the sputum film, alter the hæmoglobin and even make the medium putrefy. Care should be taken that the blood, distilled water and saponin solution are sterile. Although air contamination is not so serious as was at first thought, draughts and unnecessary exposure should be avoided. In certain techniques a source of contamination which must be prevented results from leakage of the medium or the formation of a film of moisture

extending to an unsterilised surface. Occasionally contamination may be due to an acid-resisting organism.

Proper bacteriological precautions should be taken in handling the cultures and in the disposal of the washings.

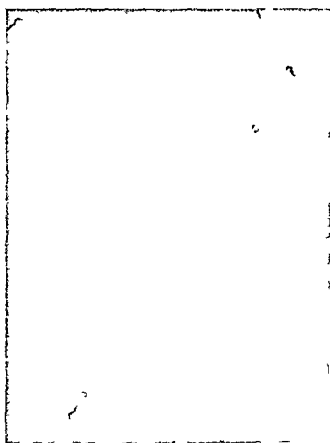
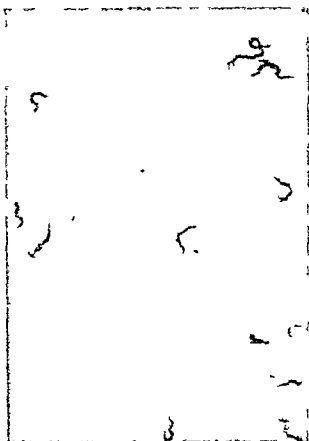
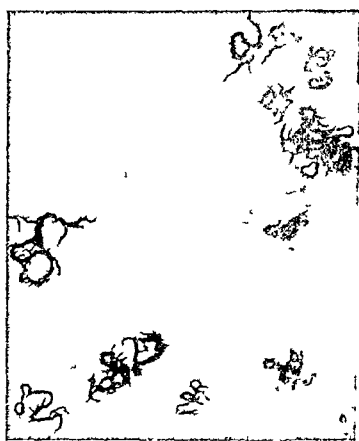
The general principle has been applied in many different ways : in slide-cells, on the floor of Petri dishes, on slides in Petri dishes, on the under surface of small slides in screw-capped bottles horizontally incubated, on slides inside vaselined bakelite, metal or glass rings and on slides in contact with hæmolysed 60 per cent. blood agar. The various methods may be classified into two groups : "open" methods, in which the medium is exposed to moist air, and "closed" methods, in which the medium is sealed up with little or no air. The open methods give better growth than the closed, but with well oxygenated blood in adequate amount this difference is minimised. Thus with ordinary slide-cells the colonies at the periphery are much larger than those in the centre, where the available oxygen is soon exhausted. But with thick cells this difference does not become apparent till incubation is prolonged more than a week. The use of an oxygen-carrying medium such as blood appears an important part of the technique, and with the closed methods of culture almost an essential part.

Of these technical variations only two will be described : the first, which was originally employed and requires no special apparatus, and a second, which can be adapted to use very little medium and which makes it easy to preserve permanent preparations of the stained cultures.

Method I. A 4-in. Petri dish is selected with a flat or slightly hollow bottom surface. The sputum is spread on this and dried. The film is treated with 15 per cent. sulphuric acid for 5 minutes. The acid is removed with a large teat and pipette, and the floor flooded with about 5 c.c. of sterile distilled water from a test-tube. This is allowed to act for a minute or so, and the washing repeated. The medium is then added and the dish placed in the incubator for a week. As medium, one can use 5 c.c. of citrated blood and either 5 c.c. of water, or better, of 1 per cent. saponin. When incubation is completed the medium is washed off with water and the film stained with Ziehl-Neelsen. Counterstaining with methylene blue may be omitted. The culture is examined under the low-power of the microscope whilst wet with water or xylol.

Method II. One or more circular patches of sputum are smeared on a glass slide and allowed to dry. A ring of bakelite, hard fibre or glass is immersed in melted vaseline and, after draining off the excess, is applied to the glass about each patch of sputum. Using a Pasteur pipette, 15 per cent. sulphuric acid is placed in the well of the ring, allowed to act for 5 minutes, and then removed. After first washing out the pipette the well is filled with distilled water. Two or three washings suffice but each change of water should be left in for a few minutes. After the final washing the medium is added—anything from 5 drops to 1 c.c. or more according to the capacity of the ring. The cultures may be incubated open to the air in a moist chamber, or closed by applying a coverslip previously immersed in vaseline.

FILM CULTURES OF TUBERCLE BACILLI

FIG 4—Original sputum film $\times 200$ FIG 5—Colonies from same sputum as in fig 4 after three days incubation $\times 200$ FIG 6—Colonies from same sputum after six days' incubation $\times 200$ FIG 7—Colonies from same sputum after eight days' incubation $\times 200$,

FILM CULTURES OF TUBERCLE BACILLI

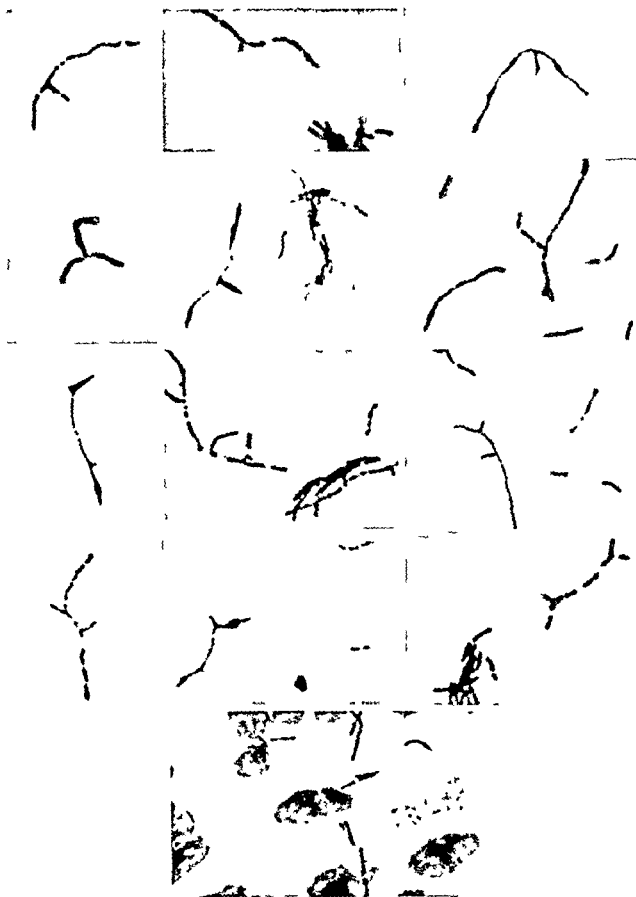


FIG. 8—Branching forms of tubercle bacilli in 6 day culture, with original sputum film below $\times 1500$

being about half the length of those in the 6-day cultures. This implies that growth does not occur throughout the length of the thread but is restricted to certain points—probably the tips. It is likely that branches take origin at secondary growth centres which develop at points where, if fission were unimpaired, the thread would have divided.

In figs. 9 and 10 are shown peculiar forms which may be identical with the "splitters" of Spengler. In the original sputum they occurred as rounded nests of rather small bacilli. These nests, which would have grown rapidly had they been ordinary clumps, were actually very little or not at all altered after 6 days' incubation. In that time the ordinary forms had grown into typical colonies.

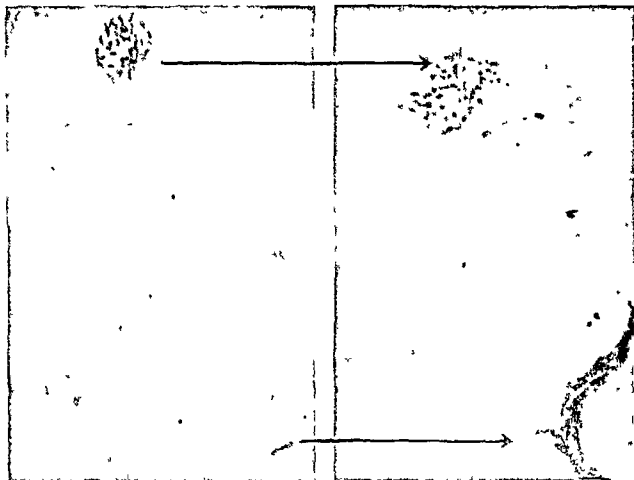
Young colonies (*i.e.* rapidly growing bacilli) stain very slightly with methylene blue, but occasionally one meets with a strain which stains deeply—chromophilic type of Pagel (1934). This is exemplified in figs. 11 and 12. All the colonies in this culture were acid-fast when decolourised for only 5 minutes, but after treating with 15 per cent. sulphuric acid for 30 minutes, many of the colonies were decolourised and subsequently stained rapidly and deeply with methylene blue. This was not just a matter of accessibility of the acid, because in thick parts of the film red colonies were often superimposed on blue colonies.

Clinical application

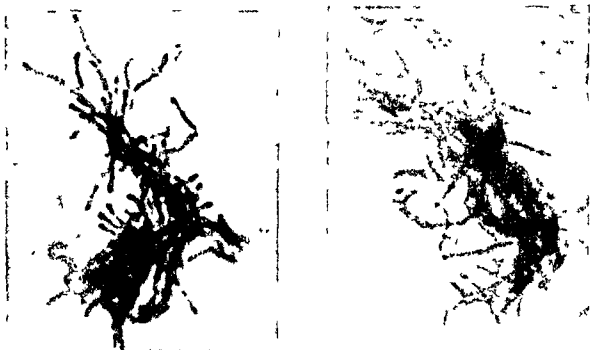
That sputum film cultures may prove useful as a diagnostic procedure is suggested by the results of an early clinical trial with sputa from 48 cases in one of the male wards at Harefield Sanatorium. In this series method I was employed, only 5 c.c. of medium (2 c.c. of blood and 3 c.c. of water) being used. Evaporation was prevented by closing the dish with adhesive tape and the use of a disc of sterilised filter paper in the roof which was moistened with sterilised distilled water. In 28 growth was luxuriant and immediately obvious. In 14 there was no growth. Of these 3 must be regarded as failures, as bacilli were found in the direct films. The other 11 showed no tubercle bacilli in the film and may be regarded as negatives. In most of these the material was salivary. In 6 cases colonies grew which were sparsely distributed. In 2 of these bacilli were present in the direct films, but in 3 cases the direct films were negative. The film of the remaining case was not available for examination.

Whilst the investigation of positive sputa will yield results of at least academic interest, what mostly concerns the clinical pathologist is the identification of tubercle bacilli from cases in which examination of direct films is negative. It takes approximately the same time (about 3 minutes) to examine 1 sq. mm. with the $\frac{1}{15}$ in. objective

FILM CULTURES OF TUBERCLE BACILLI



FIGS 9 and 10—Above, peculiar nests (? "splitters" of Spengler) in original sputum film (fig 9) and in a 6 day culture (fig 10) showing little change below, bacillus and developed colony $\times 1000$



FIGS 11 and 12—Red and blue colonies from 7 day culture, stain Ziehl Neelsen $\times 1500$

being about half the length of those in the 6-day cultures. This implies that growth does not occur throughout the length of the thread but is restricted to certain points—probably the tips. It is likely that branches take origin at secondary growth centres which develop at points where, if fission were unimpaired, the thread would have divided.

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TABLE III

Effect of barium and strontium salts on the hæmolytic activity of recalcified dialysed toxin NN849

γ $\text{SrCl}_2 \cdot 2\text{H}_2\text{O}$ added per c c of toxin	Hæmolytic effect	γ $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ added per c c of toxin	Hæmolytic effect
0 0	80	0 0	90
2 4	90	3 7	90
6 0	90	7 4	90
12 0	90	18 5	90
24 0	90	37 0	90
48 0	90	74 0	80
120 0	90	185 0	80
240 0	90	370 0	60
480 0	60	740 0	40
1200 0	40	1850 0	0

1 c c of toxin was recalcified with 80 γ CaCl_2

Diluted dialysed toxin requires relatively more calcium to enable it to hæmolyse (table II), the amount required is roughly inversely proportional to the square of the concentration of toxin. It would therefore be expected that the calcium content would have a considerable effect on the M H D of the toxin. Toxin containing effective amounts of CaCl_2 mixed with sheep red cells and allowed to stand in the cold often fails to hæmolyse, but if the red cells are centrifuged off, gently washed with cold B B S to free them from entangled toxin and then warmed with fresh B B S, hæmolysis occurs. This may mean that in the presence of ionised calcium salts toxin is readily absorbed by the cells, or alternatively that the first change in the red cell envelope is produced by toxin acting as an enzyme in the cold, the damaged cell does not set its hæmoglobin free until it is heated.

If oxalated toxin is incubated with sheep red cells for an hour and the mixture then centrifuged, the recalcified supernatant will hæmolyse sheep red cells, even those from which it has been separated, the addition of warm CaCl_2 to the separated cells does not lead to hæmolysis, showing that in the absence of ionised calcium no toxin is absorbed by the cells. Further evidence is provided by the fact that, if a determination of the M H D (see below) is made by dilution in B B S and the tubes showing no hæmolysis are centrifuged, the supernatants will, if CaCl_2 is added, hæmolyse sheep red cells, whereas such an addition to the separated cells causes little or no hæmolysis (table IV).

It might be suggested that the effect of oxalate is due not to the precipitation of calcium but to the presence of excess of oxalate. Fortunately precipitation of calcium oxalate occurs very slowly when the calcium concentration is low, and by adding red cells to oxalated toxin before precipitation is complete, it can easily be

shown that hæmolysis occurs even in the presence of considerable excess of oxalate. Similarly if oxalated toxin is recalcified with inadequate amounts of CaCl_2 and red cells are added immediately, hæmolysis occurs, even though, after precipitation of calcium is complete, addition of red cells does not lead to hæmolysis.

TABLE IV

Effect of increase in calcium concentration on absorption of α toxin by sheep red cells

Material added	Supernatants from M.H.D. determination						Material added	Separated red cells from M.H.D. determination					
	Mg. toxin originally present							Mg. toxin originally present					
	0.177	0.088	0.044	0.022	0.011	0.005		0.177	0.088	0.044	0.022	0.011	0.005
2 c.c. B.B.S.+0.5 c.c. red cells	0	0	0	0	0	0	2 c.c. B.B.S.	0	0	0	0	0	0
1 c.c. B.B.S.+1 c.c. 0.025 per cent. CaCl_2 in B.B.S.+0.5 c.c. red cells	90	90	80	60	40	20	1 c.c. B.B.S.+1 c.c. 0.025 per cent. CaCl_2 in B.B.S.	40	20	0	0	0	0
1 c.c. B.B.S.+1 c.c. 0.1 per cent. CaCl_2 in B.B.S.+0.5 c.c. red cells	90	90	90	80	40	20	1 c.c. B.B.S.+1 c.c. 0.1 per cent. CaCl_2 in B.B.S.	40	0	0	0	0	0
1 c.c. B.B.S.+1 c.c. 0.4 per cent. CaCl_2 in B.B.S.+0.5 c.c. red cells	90	90	90	80	60	15	1 c.c. B.B.S.+1 c.c. 0.4 per cent. CaCl_2 in B.B.S.	40	0	0	0	0	0
1 c.c. B.B.S.+1 c.c. 1.6 per cent. CaCl_2 in B.B.S.+0.5 c.c. red cells	90	90	90	80	60	15	1 c.c. B.B.S.+1 c.c. 1.6 per cent. CaCl_2 in B.B.S.	40	0	0	0	0	0

Diluent

Since substances which precipitate calcium salts from solution (oxalates, phosphates, fluorides) or depress their ionisation (citrates) prevent or reduce hæmolysis by α toxin, it is obvious that the M.H.D. will be greatly increased by dilution in solutions of these substances.

Prigge (1937a), finding that the M.H.D. of a number of type A toxins bore no constant relationship to the M.L.D., suggested that the toxins were composed of two different antigens, one of which was lethal (ζ = our α) and the other hæmolytic (α = our θ). For convenience we shall refer to these toxins as α and θ , transliterating Prigge's symbols as required. Later (1937b) he states that α is not hæmolytic, and that hæmolysis by type A

toxins is due entirely to their θ content. It is not therefore surprising to find that he used as diluent Jensen's buffer—a phosphate buffer of pH 7.3 which, as shown below, markedly increases the M.H.D. of α toxins. Although Prigge is certainly correct in regarding type A toxin as composed of varying amounts of two distinct antigens, his descriptions are imperfect and his reasons inaccurate. To him α is lethal, θ is hæmolytic, whereas under suitable conditions both are hæmolytic, necrotic and lethal. Hæmolysis by α toxin depends on the presence of calcium ions, which are reduced by Prigge's method of dilution, θ is lethal and necrotic, if sufficiently strong toxin can be obtained (Ipsen and Davoli, 1939), but cannot, as we have shown (unpublished observations), be titrated by injection of toxin-antitoxin mixtures intravenously into mice or intracutaneously into guinea pigs, since all mice and guinea pigs as well as a few ferrets, dogs, cats, rabbits, horses and men examined by us possessed a high concentration of circulating antibody. It is very probable that the normal antibody referred to by Ouranoff (1917) and Neill (1926) is antibody to θ toxin. Mr Glenny informs us that he is certain that the type A toxins referred to in Glenny *et al.* (1933) were antigenically pure α toxins. No difficulty has been experienced in these laboratories in producing α toxins free from θ , production of uniform θ toxins free from α has proved much more difficult.

Ipsen, Llewellyn Smith and Sordelli (1939) found that the estimated M.H.D. of a toxin later proved to be antigenically pure α toxin varied greatly in different laboratories. No information is given on the diluent used, but Miss Llewellyn Smith (private communication) informs us that she used physiological saline throughout, while Ipsen used Jensen's phosphate buffer of pH 7.3. Miss Llewellyn Smith found the M.H.D. to be 0.16 mg, while in Ipsen's laboratory the M.H.D. was 1.25 mg. It would be very interesting to know what diluent Sordelli used, for his M.H.D. was only 0.018 mg.

Penfold *et al.* (1941), attempting to concentrate gas gangrene toxins by precipitation with acetic acid, observed that toxins reconstituted in phosphate buffers or saline had very slight hæmolytic activity, though they produced hæmoglobinuria on intraperitoneal inoculation into mice, those reconstituted in broth or serum, which contain ionised calcium, were much more hæmolytic.

We have examined the effect of various diluents on the estimated M.H.D. of several α toxins. With liquid toxins the dilutions were made directly from the toxins, with dry toxins suitable amounts were weighed out and dissolved in the diluent under test.

Method. Two fold dilutions of toxin are made in volumes of 1 c.c. in Lambeth tubes of 3.4 c.c. total capacity. One half c.c. of a 6 per cent suspension of sheep red cells in B.B.S. is added to each tube and the mixture incubated at 37° C. in the water bath for one hour, allowed to cool and read about 18 hours later. The end point is taken as the faintest detectable trace of hæmolysis (15–20 per cent hæmolysis). The cell suspensions are prepared by centrifuging defibrinated sheep blood, washing twice with B.B.S. and diluting the cells to 6 per cent with fresh B.B.S.

The results for toxin NN540, which could be duplicated for many others, are given in tables V and VI. Clearly up to a point higher concentrations of CaCl_2 and MgCl_2 reduce the M.H.D., beyond that point (about 2 per cent CaCl_2 and 0.2 per cent MgCl_2) either no effect is produced or the M.H.D. is increased. Precipitants

of calcium increase the M.H.D. Normal horse serum reduces it out of all proportion to the calcium it contains (*cf.* Glenney, 1937). High concentrations of neutral salts (*e.g.* 5 per cent. NaCl) delay hæmolysis, but do not alter the M.H.D.

TABLE V

Variation of estimated M.H.D. of toxin NN540 with variation of concentration of calcium chloride in the diluent

Diluent	Mg toxin per c c diluent									
	0 71	0 355	0 177	0 088	0 044	0 022	0 011	0 005	0 0025	0 0012
Borate buffer saline (pH8)	60	60	60	20	0	0	0	0	0	0
0 003125 per cent. CaCl_2 in B B S	40	40	40	40	40	20	0	0	0	0
0 00625 " " "	60	60	60	60	60	60	20	0	0	0
0 0125 " " "	60	60	60	60	60	60	60	20	0	0
0 025 " " "	60	60	60	60	40	40	40	40	20	0
0 05 " " "	60	60	60	60	40	40	40	40	40	20
0 1 " " "	80	80	60	60	60	60	40	40	40	15
0 2 " " "	80	80	80	60	60	40	40	40	20	15
0 4 " " "	80	80	80	60	60	40	40	40	40	15
0 8 " " "	80	80	60	60	40	40	40	40	20	0
1.6 " " "	80	60	40	40	40	40	40	20	0	0

TABLE VI

Effect of various diluents on the estimated M.H.D. of toxin NN540

Diluent	Mg toxin per c c diluent												
	22 7	11 4	5 68	2 84	1 42	0 71	0 355	0 177	0 088	0 044	0 022	0 011	0 005
Borate buffer saline pH8				80	60	60	60	60	60	40	0	0	0
0 011 per cent. MgCl ₂ in B B S				80	60	60	60	60	60	40	0	0	0
0 023 " " "				80	60	60	60	60	60	40	20	0	0
0 047 " " "				80	60	60	60	60	60	60	20	0	0
0 095 " " "				80	60	60	60	60	60	60	40	20	0
0 19 " " "				80	60	60	60	60	60	60	60	40	0
0 39 " " "				80	80	80	60	60	60	60	40	15	0
0 78 " " "				80	80	80	60	60	60	60	40	15	0
1.55 " " "				80	80	80	60	60	60	60	15	0	0
3 1 " " "				80	60	40	40	20	20	15	15	0	0
6.2 " " "				80	60	40	40	40	15	15	0	0	0
M/15 phosphate-buffer-saline pH 6 0	100	90	90	60	40	15	0	0	0	0	0	0	0
M/15 phosphate-buffer saline pH 6 5	100	90	90	90	40	15	0	0	0	0	0	0	0
M/15 phosphate-buffer saline pH 7.0	100	90	90	90	80	20	0	0	0	0	0	0	0
M/15 phosphate-buffer-saline pH 7 5	100	100	90	90	80	40	0	0	0	0	0	0	0
10 per cent. normal horse serum + 0 5 per cent. tricesol				100	90	80	60	60	60	60	40	40	20

It should be remembered that, if only small amounts of α toxin are present in a mixture containing large quantities of other

haemolysins not sensitive to changes in calcium concentration, the calcium sensitivity of the α toxin may not be demonstrable until the other haemolysins are neutralised with suitable sera.

Effect of pH of diluent. A few experiments suggest that the more acid the diluent the smaller is the M.H.D., provided the diluent does not precipitate calcium. Thus if toxins are dissolved in broth at appropriate pH levels, suitably adjusted by the addition of *N*/10 HCl or *N*/10 NaOH and diluted in the same broth, the M.H.D. at pH 8 is always greater than that at pH 6.

Effect of species of red cells used as indicators

We have found that the red cells of the sheep, pig, mouse, man, guinea-pig, cow and rabbit are all about equally susceptible to the action of α toxin; goat and horse cells are practically insusceptible at the concentrations of calcium normally occurring in α toxins, but the M.H.D. for these cells can be greatly reduced by adding CaCl_2 to the toxin (table VII). θ Toxin is about equally haemolytic

TABLE VII

M.H.D. of toxin NX61 against red cells from different species

Diluent	Red cell species	Mg toxin in test												
		1 5	0 75	0 37	0 18	0 09	0 045	0 022	0 011	0 005	0 002	0 001	0 0005	0
B.B.S.	Sheep	60	60	60	40	40	40	40	40	40	0	0	0	0
0.8 per cent. CaCl_2 in B.B.S.	"	100	100	100	100	100	100	90	90	80	40	40	0	0
B.B.S.	Mouse	100	100	100	90	90	90	40	40	20	20	0	0	0
"	Man	100	100	100	90	90	90	90	90	60	40	0	0	0
"	Guinea-pig	90	90	60	60	40	40	40	40	40	0	0	0	0
"	Cat	40	40	40	40	0	0	0	0	0	0	0	0	0
"	Cow	40	40	40	40	40	20	20	0	0	0	0	0	0
"	Pig	100	100	90	90	80	80	60	60	40	40	0	0	0
"	Rabbit	100	100	90	90	60	60	40	40	0	0	0	0	0
"	Goat	0	0	0	0	0	0	0	0	0	0	0	0	0
0.8 per cent. CaCl_2 in B.B.S.	"	40	40	40	40	40	40	40	40	40	40	40	0	0
B.B.S.	Horse	40	0	0	0	0	0	0	0	0	0	0	0	0
0.8 per cent. CaCl_2 in B.B.S.	"	80	80	80	80	80	80	40	40	20	20	0	0	0

for the red cells of all species tested, except those of the mouse, which are only slightly attacked. δ Toxin produces no effect on any red cells examined except those of the sheep, pig, cow and goat—all even-toed ungulates (*Artiodactyla*). Work on the chemical factors determining this specificity would be of great value.

Effect of peptone

There is a striking difference in the rate of haemolysis of sheep red cells by different α toxins. In our experience toxins produced during relatively short periods of incubation of the culture are

much more rapidly hæmolytic than "long growth" toxins. Thus 1 c.c. of a toxin (NX60) obtained from a culture incubated for $2\frac{1}{2}$ hrs. at 37° C. hæmolyzed 0.5 c.c. of a 6 per cent. suspension of cells in $1\frac{1}{2}$ minutes at 37° C., though its combining power was only 0.6 α units; 1 c.c. of a toxin (NX55A) from an 8-hour culture had not caused complete hæmolysis after 1 hr. at 37° C., though its combining power was 1.8 α units. Most though not all of this difference is due to differences in peptone concentration, for if peptone is added to slowly hæmolytic toxins, the hæmolytic time is greatly reduced. Thus by adding 1 per cent. Parke Davis peptone to NX55A the time taken to produce hæmolysis is reduced to 3 minutes. Examination of the nitrogen distribution in long- and short-growth toxins shows clearly the progressive breakdown of the peptone and proteose fraction and its partial exhaustion after a relatively short period of incubation (table VIII).

TABLE VIII
Nitrogen distribution in α toxins

	Original broth	Toxin NX60A	Toxin NX55A
Time of incubation . . .	0	$2\frac{1}{2}$ hrs.	8 hrs.
Total nitrogen per 10 c.c. .	43.0 mg.	43.4 mg.	47.4 mg.
"Copper * nitrogen" per 10 c.c.	7.64 mg. (17.8 per cent.)	10.0 mg. (23 per cent.)	14.73 mg. (31.1 per cent.)
Proteose and peptone nitrogen per 10 c.c.	22.9 mg.	16.1 mg.	6.03 mg.
"Copper nitrogen" per 10 c.c. .	2.08 mg. (9.1 per cent.)	2.5 mg. (15.5 per cent.)	1.25 mg. (20.7 per cent.)

* Pope and Stevens (1939)

Addition of peptone does not alter the combining power, though it may reduce the M.H.D. somewhat. Similar observations on the reduction of the hæmolytic time have been made by Llewellyn Smith and Price (1938) on the β toxin of the staphylococcus.

For routine purposes it is usual in these laboratories to dissolve α toxin in broth containing 1 per cent. of Parke Davis peptone. It is probable that the original reason for this custom was the buffering effect of broth on unstable toxins; if broth is used as the diluent the end-point is sharper and the results are more consistent. Moreover the calcium and peptone in the broth reduce the test dose and hasten hæmolysis, thereby economising both toxin and time.

OPACIFICATION OF SERUM

Seiffert (1939) and Nagler (1939) independently observed that the addition of type A toxin to human serum led to the production of opacity. Seiffert claimed that this opacification was not prevented

by α antisera, but Nagler (1939) and Macfarlane *et al* both found that α antisera could be titrated against α toxin, using human serum as indicator. Seiffert states that the opalescence is produced with human sera only, Nagler added fowl sera and the serum of one monkey. We found that opacity was produced by α toxin in the sera of 9 horses, 5 ferrets, 10 guinea pigs, 3 rabbits, 1 sheep, 2 mice and 10 dogs, and that this opalescence was prevented by the previous addition of adequate amounts of α antitoxin. Sera repeatedly frozen and thawed give poor opacity, presumably owing to removal of free fat. The minimal indicating dose is reduced by the addition of calcium salts (up to 2 per cent) to the diluent, and the opacity is prevented by oxalates and citrates.

OPACIFICATION OF LECITHOVITELLIN (L V)

Macfarlane *et al* suggested the use of this indicator for titrating α antisera, and showed that the effect of α toxin on it is dependent on the presence of ionised calcium. We have found that increase of calcium salts up to 2 per cent in the diluent reduces the minimal opalescing dose, still further addition increases it. It might be supposed that, as L V becomes opalescent with traces of toxin which are without apparent effect on red cells, it combines readily with smaller amounts of toxin than do red cells. This is partly true, for L V contains amounts of calcium salts which facilitate reaction with toxin, whereas our red cell suspensions do not, but the difference between the minimal opalescing dose and the M H D is far greater than could be accounted for by the calcium alone. Far more of the effect is due to the fact that it is easier to detect traces of opalescence than it is to detect traces of hæmolysis. Thus if 0.5 c.c. of 6 per cent sheep red cells is completely hæmolysed with distilled water, no pink colour can be detected at a dilution of 1:128, whereas if a batch of L V be treated with an amount of toxin just sufficient to aggregate the fat completely, and the mixture so produced is diluted in unaltered L V, opalescence can be observed to a dilution of 1:1000 or more. If therefore a sample of toxin produced a change of equal magnitude in red cells and L V, say $\frac{1}{1000}$ of complete hæmolysis or aggregation, the change in L V could be observed while the hæmolysis could not.

LETHAL ACTION

The M L D, as judged by the injection of 0.5 c.c. of α toxin intravenously into mice, is unaffected by the nature of the diluent so long as the diluent alone is not injurious to mice. Addition of calcium salts up to 0.4 per cent CaCl_2 makes no difference to the M L D, presumably because the calcium ions diffuse from the circulation more rapidly than the toxin.

NECROTISING EFFECT

The lesions produced in the skin of guinea-pigs by the intracutaneous injection of α toxin are described by Glenny *et al.* (1931). The M.N.D. is not affected by addition of CaCl_2 , or by harmless diluents.

COMBINING POWER OF α TOXIN

We have carried out determinations of the combining power of a number of α toxins at various levels against several α antitoxins, using four different indicators, (1) red cells, (2) lecithovitellin, (3) lethality to mice and (4) flocculation. For convenience we shall refer to the α antitoxic values obtained by titrating a serum against the test dose of an α toxin using a particular indicator as the hæmolytic, L.V., lethal or flocculating value of the serum. Hæmolysis, opacification of lecithovitellin and lethality differ markedly from flocculation in that by them free toxin is determined, whereas in flocculation the tube flocculating first is supposed to be neutral. Consequently any method which reduces the M.H.D., for instance, should increase the combining power, though not necessarily very much, since traces of toxin otherwise undetermined will thereby be demonstrated. Flocculation on the other hand should be unaffected. The ultimate standard of reference throughout has been British standard gas gangrene serum (*perfringens*) containing 20 international units per c.c. The current standards were G.G.C. 3089 (475 α units per c.c.), G.G.C. 3768 (850 α units per c.c.) and R.X. 5102 (10 α units per c.c.).

Hæmolytic testing

We have given most attention to the effect of CaCl_2 on combining power. The percentage of calcium in the toxin was determined by precipitation with ammonium oxalate at pH 8 and titration of the washed precipitate with standardised permanganate. The L_h equivalent to 1 α unit was determined and various multiples and sub-multiples of this were used as test doses for different levels of testing against convenient sera of known composition at varying concentrations of CaCl_2 , making allowance for the calcium content of the original toxin. As we have no knowledge of the condition in which the calcium of the toxin is combined, except that some of it must be ionised, we express the total concentration of calcium salts as the molar concentration of calcium in the total test volume. For the purposes of this paper 1 *M.* (Ca) = 40.07 g. calcium per l.

Method. Dissolve dry toxin in B.B.S. to give double the concentration to be used in the test; dilute convenient volumes of this solution with equal volumes of B.B.S. containing varying concentrations of CaCl_2 ; we have used 0.003125, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 per cent. To the test dose of toxin used add varying volumes of antitoxin differing by

convenient amounts (usually 10 per cent), and make up to constant volume with BBS. Allow to stand for 30 minutes, add 0.5 c.c. of 6 per cent sheep red cells, incubate for 1 hour in the water bath with the water level 1 cm. or more below the level of the mixture in the tubes, allow to cool and read after about 18 hours. The end point is taken as the faintest detectable trace of hemolysis (15.20 per cent). The error of a single test is less than 10 per cent, i.e. the value obtained from this single test will, 19 times out of 20, not differ by more than 10 per cent from the mean of a large number of observations. From the values obtained a graph is constructed showing the relationship between log calcium concentration and log combining power of the test dose, and to these points a 'best straight line' is fitted by the method of least squares. No curve is based on less than 10 points. For reasons to be stated later we call these 'calcium avidity curves'.

Altogether 53 different toxin-antitoxin systems have been examined. The results can best be seen from the graphs (figs 1-4) which show that at any given test dose there is a steady increase of estimated combining power with increasing concentration of calcium salts, the relationship is clearly logarithmic and the correlation very high. More striking is the fact that the curves for toxin NX22A and serum R 5603 at different test doses are not parallel, the angle with the horizontal is greater the smaller the test dose of toxin (fig 1). The difference in angle for successive decreases in test dose is not very great, but the values for the tangent of the angle (m) are in linear order of increase, the odds against this occurring by chance are 720 to 1. This suggested that the reduction in the M.H.D. produced by increased concentration of calcium salts might not be the only factor in altering the estimated combining power of the toxin but that the calcium salts might in some way lead to the dissociation of the toxin-antitoxin complex and thereby increase the amount of antitoxin necessary for neutralisation.

If we suppose that, when toxin and antitoxin are mixed, combination occurs according to the law of mass action, it follows that, whatever the nature of the reaction, combination can never be complete, but an equilibrium will be approached between toxin, antitoxin and complex. The higher the original concentration of antitoxin the lower will be the equilibrium concentration of toxin. Our determination of neutrality must therefore involve the estimation of a certain minimum of free toxin or antitoxin, and since we have no means of determining antitoxin except by its capacity to neutralise toxin, we are in practice limited to determining free toxin. As no physical method yet exists for this purpose, the presence or absence of free toxin is demonstrated by adding an indicator, e.g. susceptible red cells, human serum or lecithovitellin, or by the injection of the reaction mixture intravenously into mice or intracutaneously into guinea-pigs. These indicators can show the presence of toxin only by combining with it. If no calcium ions

are present, no combination of toxin and indicator occurs, no toxin can be demonstrated and, however much free toxin is present, the mixture will appear to be neutral or overneutralised. Since no calcium-free laboratory animal is available, this desirable state of

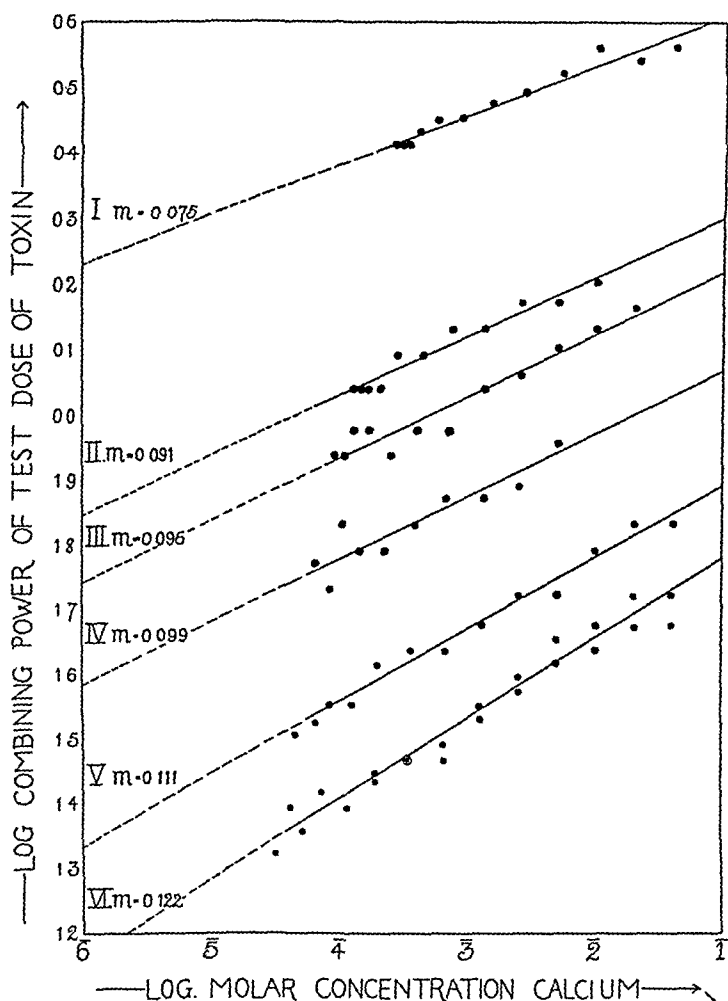


FIG. 1.—Calcium avidity curves for toxin NX22A and serum R. 5603 at varying L_h levels, I at $2 L_h$, II at L_h , III at $0.7 L_h$, IV at $0.5 L_h$, V at $0.35 L_h$, VI at $0.25 L_h$. m = the tangent of the angle between the best straight line and the abscissa. The ringed point represents two equal determinations. (Hæmolytic testing.)

affairs cannot be realised *in vivo*. In the presence of calcium ions toxin and indicator combine and toxin is removed from the system at a rate dependent on the concentration of toxin and calcium ions; in consequence the equilibrium of the system is disturbed, and to restore it the toxin-antitoxin complex must dissociate. The higher

the concentration of calcium ions, the more toxin will be removed from the system in a given time, the greater will be the disturbance of the equilibrium, the more complete the dissociation of the complex and the greater the effect on the indicator. The only way in which

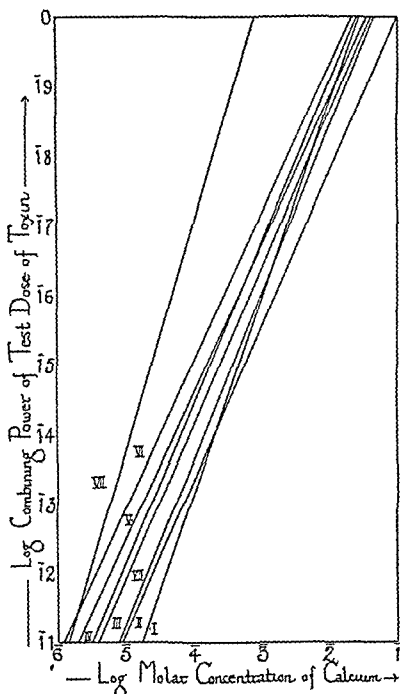


FIG. 2.—Calcium acidity curves for toxin NN540 and various sera I R 5785 II, GGC 3768 III, GWP 160 F 36 IV, R 6891 V, R 6932 VI, R 6918 VII, S 602 VIII, R 5603 (Hæmolytic testing) The dot lies between the Roman numeral and the line referred to To avoid confusion no points are plotted in this and the remaining graphs, the correlation coefficient is seldom less than 0.8

dissociation of the complex can be repressed without effect on the indicator is to add antitoxin, the more antitoxin there is added the less dissociation will occur, until a point is reached at which the toxin available to the indicator in the course of the experiment is reduced below the minimal indicating dose This is the neutral

mixture for the particular circumstances and will in general indicate a higher combining power for the test dose of the toxin the higher the concentration of calcium ions. At lower test doses of the toxin, combination will naturally be slower, relatively larger amounts of

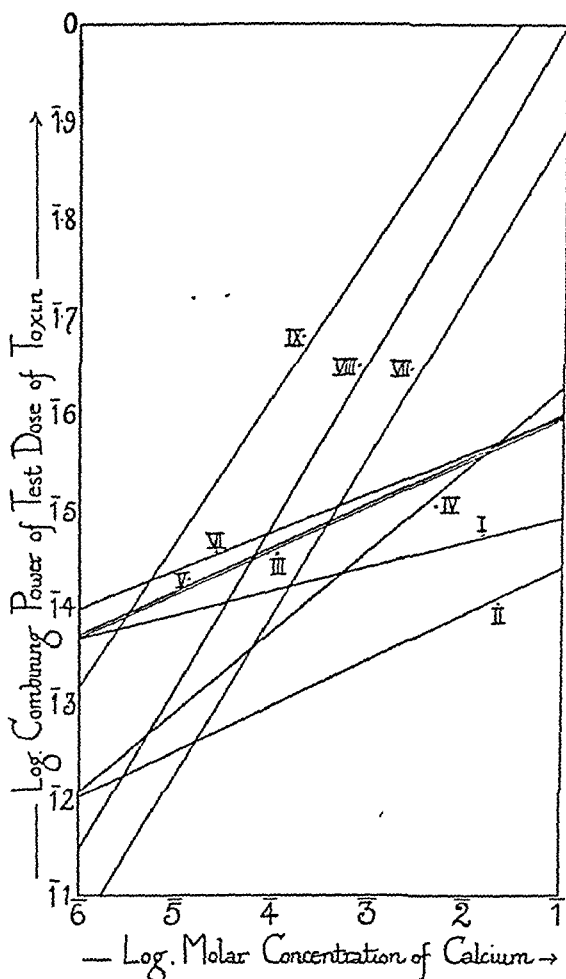


FIG. 3.—Calcium avidity curves for toxin NX118 and various sera. I, R. 6891. II, G.G.C. 3768. III, G.W.P. 160.F.36. IV, R. 5785. V, R. 5603. VI, R. 6932. VII, S. 602. VIII, S. 601. IX, S. 600. S. 600, 601 and 602 are irregular. (Hemolytic testing.)

toxin will remain free at the time the indicator is added and relatively more antitoxin will have to be added to repress the dissociation of the complex as the toxin is removed by the indicator. The view that competition between indicator and antitoxin for toxin is the reason for these findings is, we think, supported by the observation that the same values are obtained at a given test dose

of toxin for the combining power at varying calcium levels whether the calcium is added to the system before or after the toxin has been in contact with antitoxin. In all this there is of course nothing new except the fortunate circumstance that in α toxin-antitoxin titrations we can vary at will the rate of combination of toxin and indicator in a quantitative manner.

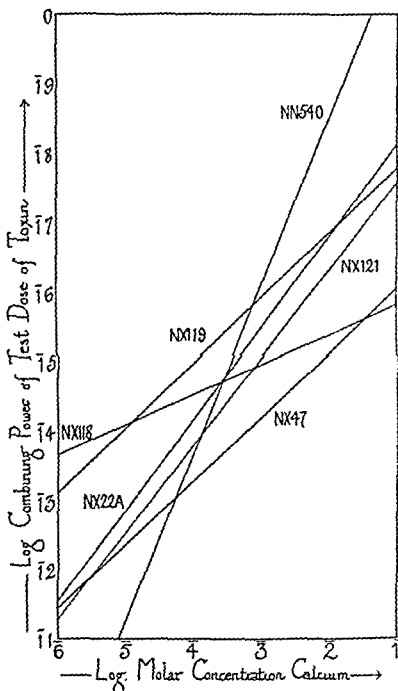


FIG. 4.—Calcium avidity curves for serum G.W.P. 160.F.36 and various toxins.
(Haemolytic testing.)

This view assumes that, when hæmolysis for example is produced by α toxin, toxin is removed from the system; this is supported by the fact that dilutions of α toxin which have hæmolysed red cells produce much less effect on lecithovitellin than similar dilutions of toxin to which red cells have not been added. Judged by this test, both laked blood and stromata absorb α toxin. Further confirmation of the view that the apparent increase in combining

power with increase in the concentration of calcium is due to the dissociation of the toxin-antitoxin complex is obtained from experiments with other α toxins and sera (figs. 4, 5). The curves at a level of 0.25 L_h at varying concentrations of calcium salt for different toxins and sera are not parallel, suggesting that the

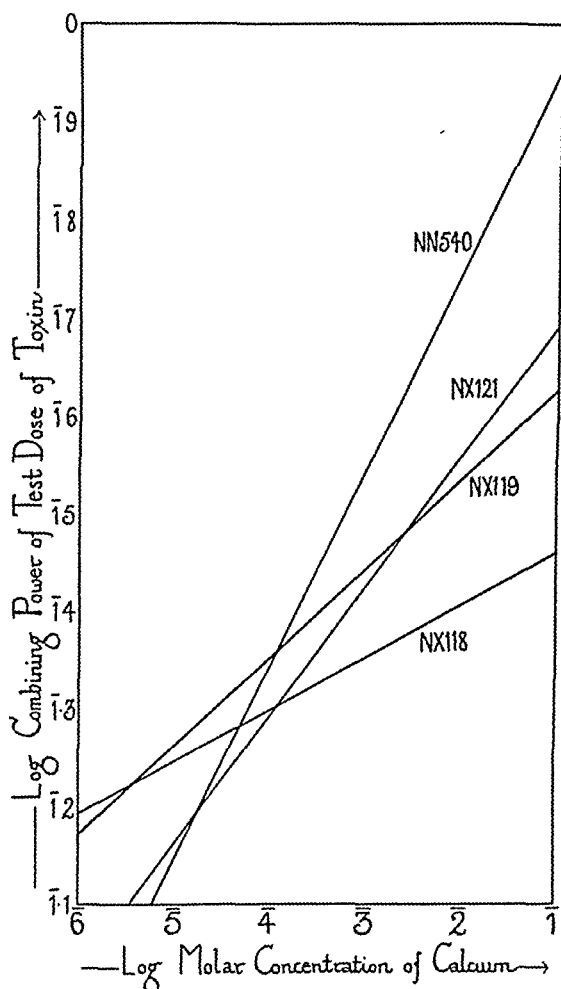


FIG. 5.—Calcium avidity curves for serum G.G.C. 3768 and various toxins. (Hæmolytic testing.)

complex is not equally stable for all systems. It will be noted that a serum which is easily dissociated from one toxin is not so easily dissociated from another, *cf.* serum G.W.P. 160.F.36, which dissociated with difficulty from toxin NX47 but easily from toxin NN540 (fig. 4). Clearly this is another example of "avidity"; but since the dissociation of the toxin-antitoxin complex is not entirely dependent on the serum, it might be better to speak of the

avidity of the constituents of the complex rather than of the serum as is usually done

It might be supposed that, if the complex were allowed to flocculate out of a set of toxin-antitoxin mixtures prepared for a combining power test and the floccules removed, as less of the complex is left to dissociate, the effect of the addition of CaCl_2 on the estimated combining power of the toxin would be less than if flocculation had not occurred. Fig. 6 shows that this is so. Moreover, if the floccules are washed with BBS and the centrifuged washings or floccules treated with calcium salts, hæmolytic may be produced when red cells are added

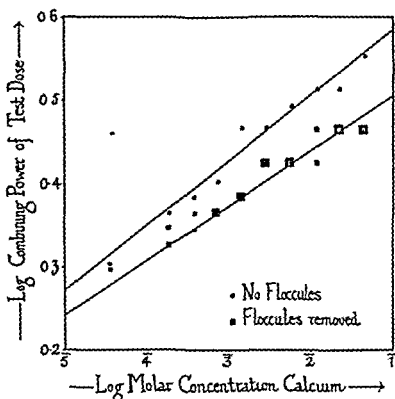


FIG. 6.—Calcium avidity curves of toxin NX47 and serum GWP 160 F 36 with and without flocculating out (Hæmolytic testing). The large squares represent two equal determinations.

That the complex is readily dissociable by simple procedures is shown by the observation of Ipsen, Llewellyn Smith and Sordelli (1939) that the estimated combining power of α toxin is increased by cooling the mixtures after incubation with red cells. They suggest that this change is due to dissociation on cooling. Two years ago Miss H. E. Ross informed us that, when she was in a hurry to obtain the results of an α titration, she usually cooled the tubes under a cold water tap. We had previously been impressed by the rapidity with which free α toxin is absorbed by red cells, we therefore carried out the following experiment.

To a series of tubes containing the test dose of toxin, quantities of antitoxin differing by 10 per cent. were added and the volume made up with BBS. The mixtures were allowed to stand for 30 minutes, 0.5 cc. of

6 per cent. sheep red cells was added to each tube and the sets were incubated for 1 hour at 37° C. They were then removed from the bath and centrifuged rapidly in warm cotton wool. The supernatants were pipetted off and added to new red cells, while the separated red cells were lightly washed and resuspended in warm B.B.S., incubated for a further 30 minutes and allowed to cool. The tubes were arranged in the rack according to the amounts of antitoxin, the highest antitoxin levels being on the left. As was expected the supernatants showed hæmolysis in tubes further to the left than did the resuspended red cells. Assuming that any free toxin is readily absorbed by red cells, the only possible explanation is that the toxin-antitoxin complex had dissociated on cooling.

Since flocculation tests are self-indicating and there is no competition between indicator and antitoxin for toxin, changes in calcium concentration would not be expected to produce any effect on the estimation of the neutral mixture. Within the limits of experimental error this is true. Moreover, within the range 10-1 α over which this method is practicable, sub-multiples of the test dose measure corresponding sub-multiples of combining power, a result which would hardly be expected if ionised calcium were involved.

Just as increase in the concentration of calcium salts raises the estimated combining power of the test dose of an α toxin, so addition of oxalate or citrate reduces it. The only condition in which this is likely to arise in practice is in the testing of the serum or plasma of normal horses for α antitoxin in order to determine their usefulness for immunisation. In such testing, two-fold dilutions of the test dose of the toxin (at 1 α unit) are mixed with 0.5 c.c. of undiluted horse serum. If oxalated plasma is used instead of serum the lower dilutions of toxin will be unable to combine with the indicator and the plasma will appear to contain a higher concentration of α antitoxin than the serum. Thus two consecutive bleedings from horse 9582, one of 40 c.c. into 10 c.c. of B.B.S. and the second of 40 c.c. into 10 c.c. of 1 per cent. oxalate, gave serum $\frac{1}{200} \alpha$ per c.c., plasma $\frac{1}{8} \alpha$ per c.c.

In a few experiments the effect of ionised magnesium salts on the combining power of α toxin was similar but much smaller and the difference at different levels much more obvious (fig. 7). The fit of the points is noticeably poorer. Experiments on the effect of increasing calcium concentration on the combining power of liquid toxins have not been practicable, as the addition of B.B.S. to toxins so altered leads to the precipitation of calcium phosphate. Experiments on the effect of increasing concentrations of barium and strontium on dry toxins have given very inconsistent results, presumably owing to the precipitation of barium and strontium sulphates by the traces of ammonium sulphate which it is impossible to remove from the toxin. Attempts have been made to use the number of M.H.D. per L_h as an indication of the nature of the toxin

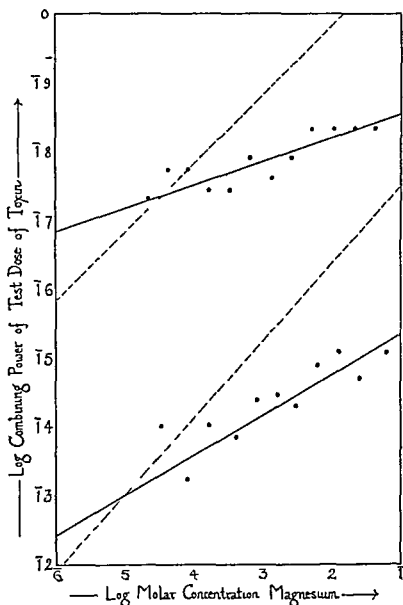


FIG 7—Magnesium avidity curves for toxin NX22A and serum R 5603 at 0.25 and 0.5 L_h . Interrupted line = corresponding calcium curve (Hæmolytic testing)

TABLE IX

Values of $L_h/4$ and M H D at different calcium concentrations

Molar concentration of calcium	$L_h/4$ (mg)	M H D (mg)	$\frac{L_h/4}{M H D}$
8.1×10^{-6}	13.59	0.085	160
2.89×10^{-4}	6.38	0.010	640
5.7×10^{-4}	5.47	0.010	550
1.13×10^{-3}	4.69	0.005	940
2.26×10^{-3}	4.03	0.0025	1610
4.50×10^{-3}	3.47	0.00125	2890
9.00×10^{-3}	2.98	0.00125	2490
1.80×10^{-2}	2.56	0.00125	2050
3.60×10^{-2}	2.21	0.00125	1770
7.19×10^{-2}	1.88	0.0025	750
1.44×10^{-1}	1.57	0.005	310

under test or of the amount of toxoid present. Table IX, which gives the value of this ratio for toxin NN540 against serum

G.G.C. 3768, shows that any judgments based on this ratio for an α toxin rest on a very precarious foundation, unless the calcium content of the toxin is taken into consideration. For instance, at 8.1×10^{-6} M (Ca) the number of M.H.D. per $L_h/4$ is only 160; at 4.5×10^{-3} M (Ca) it is 2890.

Addition of normal horse serum to toxin-antitoxin mixtures in hæmolytic tests leads to an increase in the estimated combining power of the toxin, probably due to the calcium content of the serum (table X).

TABLE X

Combining power of varying test doses of toxin NX60 dissolved in various diluents against serum R. 5603

Test dose (L_h)	Diluted in B.B.S.		Diluted in B.B.S. + 2 per cent. horse serum	Diluted in 0.8 per cent. CaCl_2 in B.B.S.	
	Hæmolytic testing	L.V. testing	Hæmolytic testing	Hæmolytic testing	L.V. testing
2.0	2.0 α	2.5 α	...	2.72 α	2.72 α
1.0	1.0 α	1.41 α	1.36 α	1.66 α	1.66 α
0.5	0.45 α	0.68 α	0.66 α	1.1 α	0.86 α
0.2	0.12 α	0.29 α	0.26 α	0.36 α	0.33 α
0.1	0.064 α	0.1 α	0.12 α	0.19 α	0.18 α
0.05	Failure	0.05 α	0.056 α	0.12 α	0.078 α
0.02	0	0.018 α	0.024 α	0.05 α	0.032 α
0.01	0	0.009 α	0.012 α	0.033 α	0.02 α
0.005	0	0.0045 α	Failure	0.018 α	0.006 α
0.002	0	Failure	Failure	Failure	Failure

"Failure" = no consistent result obtained.

Comparison of hæmolytic with other forms of testing

Opacification of serum

Macfarlane *et al.* showed that α antitoxin could be titrated using human serum or lecithovitellin as indicator. It is often necessary when testing at low levels of α , as in normal horses, to use undiluted serum, and the question naturally arose whether the serum under test could be used as its own indicator.

To two-fold B.B.S. dilutions of the L_h of α toxin were added 0.5 c.c. amounts of undiluted horse serum filtered free of suspended debris. The mixtures were allowed to stand for half-an-hour, then incubated for 2 hours at 37°C . Opalescence appeared in mixtures which were underneutralised as judged by corresponding hæmolytic tests. Horse sera of known high α value diluted in normal horse serum of negligible anti- α content showed good agreement with expectation when titrated by this method, which is, however, too troublesome for routine use.

Opacification of lecithovitellin

Method. The L_h dose of toxin or a convenient sub-multiple is measured into Lambeth tubes and volumes of serum differing by 10 per cent. added. After the tubes have stood for 30 minutes at room temperature 0.5 c.c. of crude lecithovitellin suspension is added and mixed by inversion. The tubes are then incubated for 1 hour at 37°C . If time is pressing, readings may be taken immediately; for delicate work it is better to wait till next day. The crude end-point is taken as that tube which, by ordinary inspection in the rack, appears more opalescent than its nearest neighbour containing more antitoxin. Careful comparison with proper controls usually shows that tubes containing more serum than the crude end-point tube are slightly opalescent, showing either that there is a long "trail" of traces of toxin or that lecithovitellin can produce slight dissociation of the toxin antitoxin complex, even in the presence of excess antitoxin.

Comparative tests of the combining power of α toxins have been carried out at various test doses using red cells and lecithovitellin as

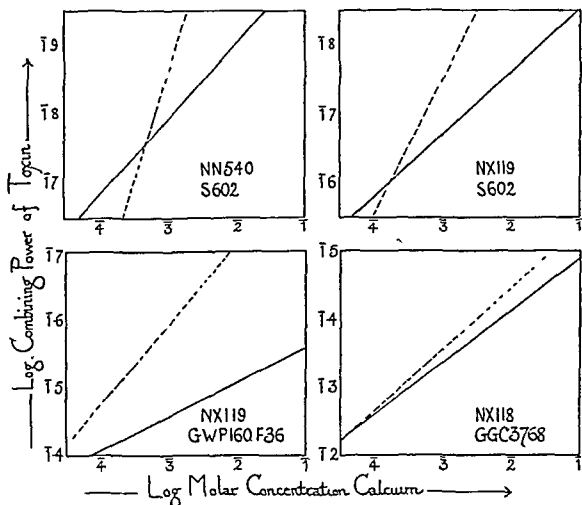


FIG. 8.—Calcium avidity curves for various toxins and sera by L.V. testing. Interrupted line = corresponding hæmolytic curve. As the hæmolytic and L.V. curves were done at a considerable interval, no special notice should be taken of the absolute values of the combining power. The values of $m_{L.V.}$ are probably as accurate as can be obtained over the restricted range available for test.

indicators; if the toxin is diluted in B.B.S., lecithovitellin tests always indicate a higher combining power for a given test dose than do hæmolytic tests, presumably because lecithovitellin

suspensions contain calcium equivalent to $2.3 \times 10^{-3} M$, whereas our red cell suspensions contain no calcium effective for hæmolysis. It is partly for this reason that the addition of calcium salts produces less increase in the estimated combining power of a toxin in L.V. tests than in hæmolytic tests; the relative increase in calcium concentration is less. As would be expected the greater the dilution in B.B.S. the greater the L.V./hæmolytic ratio for the combining power of the toxin (table X).

We have examined the effect of increased calcium concentration on the combining power of α toxins, using L.V. as indicator, in

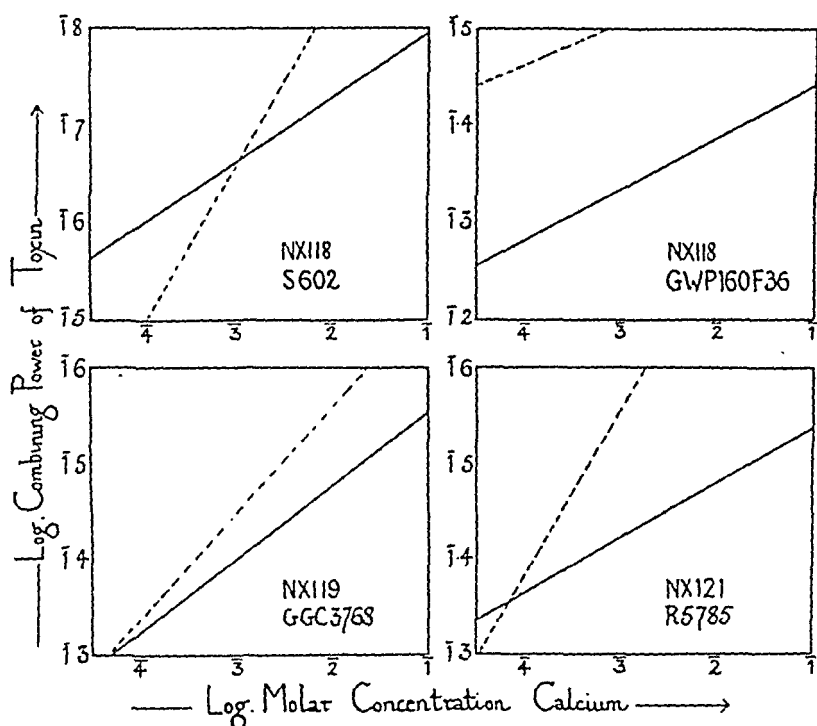


FIG. 9.—Calcium avidity curves for various toxins and sera by L.V. testing. Interrupted line = corresponding hæmolytic curve.

much the same way as we did with hæmolytic tests. Twelve toxin-antitoxin systems have been examined by this method. Owing to the high calcium content of the L.V. the calcium range is much restricted and the best straight line correspondingly less accurate; it seems clear that increased calcium concentration leads to increase in estimated combining power though less than is indicated by hæmolytic tests (figs. 8 and 9). Consequently if determinations of the α antitoxic value of a serum are carried out in parallel at the same test dose, using red cells and L.V. as indicators, the L.V. test will indicate a lower value than the hæmolytic test, though the

effect will be rather less than would be expected from the effect of calcium on hæmolytic tests. It seems necessary to assume that L V removes less toxin from the system than do red cells. Toxin which has affected L V seems no less hæmolytic than before, though, of course, the calcium in the added L V increases the hæmolytic effect.

Mr A T Glenny early drew our attention to the fact that if hæmolytic and L V tests were carried out in parallel at the same test dose, the discrepancy between the serum values so obtained was between 0 and 10 per cent for a liquid toxin, while for a dry toxin it was often 10-20 per cent, in both cases the L V value was the lower. Since dry toxins contain very little calcium, the effect of the addition of L V in raising the calcium concentration would naturally be much greater in dry than in liquid toxins, in which the calcium concentration is usually much higher (table XI). Since

TABLE XI

Molar concentration of calcium in various preparations

Preparation	Molar concentration of Ca
Dry toxin (NN540)	2.4×10^{-4}
Liquid toxin (NN907)	48.0×10^{-4}
Lecithovitellin (L V)	26.9×10^{-4}
1 cc dry toxin + 0.5 cc L V	10.6×10^{-4}
1 cc liquid toxin + 0.5 cc L V	40.96×10^{-4}
1 cc dry toxin + 0.5 cc rbc suspension	1.5×10^{-4}
1 cc liquid toxin + 0.5 cc rbc suspension	32.0×10^{-4}

the greater the change in calcium concentration the greater will be the increase in combining power and the lower naturally will be the corresponding estimation of the serum value.

An interesting point is the striking accuracy of L V testing at low levels, with hæmolytic testing it is seldom possible to test below $\frac{1}{10} \alpha$ unit with an accuracy of 10 per cent, with L V testing an accuracy of 10 per cent can readily be obtained from $\frac{1}{10}$ to $\frac{1}{200} \alpha$ unit.

As previously observed, it is often necessary to test for α anti-toxin in undiluted sera, since high concentrations of sera prevent aggregation of fat in lecithovitellin altered by α toxin, it was at first thought that titration of normal horse sera by this method would be impracticable. Further examination of such reaction systems showed, however, that faint opalescence is still produced in underneutralised mixtures and by careful comparison with suitable controls results corresponding very well with those found by hæmolytic testing could be obtained. Although it is possible to test at very low levels (e.g. $\frac{1}{1000} \alpha$), the case with which hæmolytic

tests can be carried out down to $\frac{1}{100} \alpha$ makes the hæmolytic method preferable unless the serum is markedly hæmolytic to sheep red cells.

Lethality

As would be expected from the free diffusibility of calcium ions, increased concentration of calcium salts makes no difference to the combining power of α toxins as judged by intravenous injection into mice. Since we can by suitable methods vary the ratio between the L_h and L_+ for a given toxin, it is clear that the fact that L_h and L_+ are in constant ratio for the great majority of α toxins is no adequate proof that hæmolysis and lethality are produced by the same antigen (table XII). For complete serological proof it is

TABLE XII

Effect of calcium concentration on the ratio L_+/L_h

Toxin	Molar concentration of calcium	L_h (mg.)	L_+ (mg.)	$\frac{L_+}{L_h}$
NX61	4.1×10^{-4}	5.0	5.5	1.1
	3.6×10^{-2}	3.4	5.5	1.62

necessary to show that, if a serum is titrated against a standard, the hæmolytic and lethal values of the serum against the same α toxin are equal within the limits of experimental error. This has been done by Glenney *et al.* (1933).

A few experiments on the intracutaneous test in guinea-pigs have shown that the combining power as measured by this method is unaffected by the calcium concentration in the reaction mixture.

Flocculation

Flocculation occurs readily in suitable mixtures of dry or liquid α toxins and fresh antitoxins. At 2 α units the flocculation time is about 90 minutes at 50° C.; the error is about 10 per cent. in one test. No concentration of the toxin is necessary. Refined sera (Pope, 1939) flocculate much more readily than crude or concentrated sera. Neither the flocculation time nor the flocculation value of the sera is affected by the calcium concentration.

Irregular sera

For the great majority of α antisera the hæmolytic is seldom more than 20 per cent. greater than the L.V. value. Some sera, however, consistently give the L.V. values 30-50 per cent. lower than the hæmolytic; these we call irregular sera. We have already shown that the relationship between log. calcium concentration and log. combining power for any particular toxin and antitoxin

can be expressed with considerable accuracy by a straight line, whose slope can as usual be represented by the tangent of the angle it makes with the horizontal. This value (m) is fairly constant for any given toxin, though different toxins vary very much among themselves. Though there appears to be no exact relationship between the values of m obtained by hæmolytic and L.V. testing (m_H and m_{LV}) for the same system, when m_H is high, m_{LV} is also usually high. Since the error introduced by assuming a

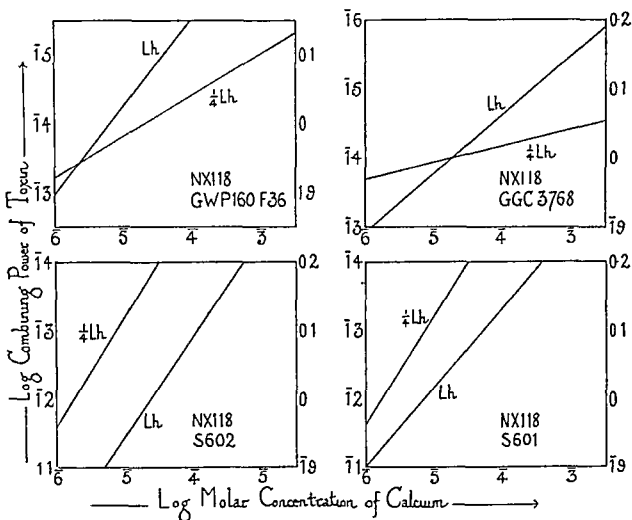


FIG. 10.—Calcium avidity curves for toxin NX118 and two normal (above) and two irregular sera (below) at different levels. Figures on left of blocks refer to testing at $\frac{1}{4}$ Lh, those on right to testing at 1 Lh.

constant difference between m_H and m_{LV} is not great, the argument following will be simplified by this approximation. If two different sera are compared as to their α antitoxin content against the same toxin, the L.V. and hæmolytic values will show the same difference for both if the values of m_H (and therefore of m_{LV}) for both systems are about the same. But if these are different the differences between the hæmolytic and L.V. values will not be of the same order; the higher the value of m_H (and therefore of m_{LV}), the greater will be the difference between the hæmolytic and L.V. values of the serum. We therefore supposed that the irregular

sera would show calcium avidity curves of steeper slope than is usual for the toxin under test; the calcium salts in the L.V. would produce an unusually large increase in the combining power of the toxin and correspondingly greatly reduce the L.V. value of the serum. Figs. 2 and 3 show that three irregular sera examined by us (S. 600, S. 601, S. 602) all show values of m_H unusually high for the toxins against which they were tested; this applies to all four toxins used. The difference occasionally observed between the values for the same serum against dry and liquid toxins tested at

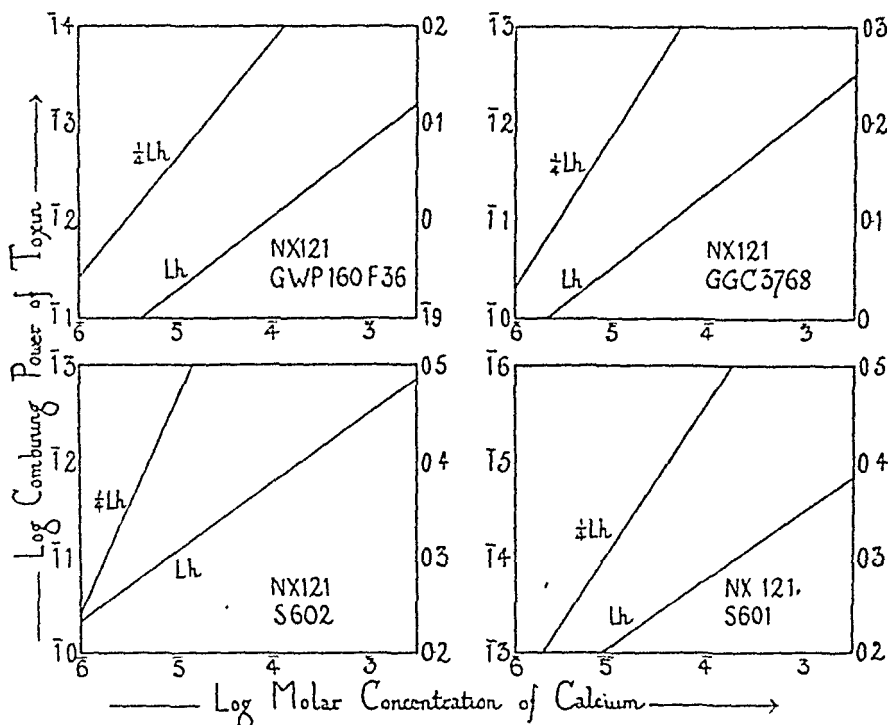


FIG. 11.—Calcium avidity curves for toxin NX121, as in fig. 10.

the same level is probably also due to the serum being irregular and its toxin-antitoxin complex therefore affected to an unusual degree by the excess of calcium in the liquid toxin. We also supposed that irregular sera would be non-avid in hæmolytic tests at constant calcium concentration and in animal tests as compared with normally behaving sera. The results of hæmolytic tests are shown in figs. 10 and 11. It appears that if the test toxin (e.g. NX121, fig. 11) ordinarily gives a steep calcium avidity curve at $\frac{1}{4}$ L_h, the curve at 1 L_h for both regular and irregular sera will be less steep than that at $\frac{1}{4}$ L_h; i.e. both kinds of sera appear non-avid. With test toxins (e.g. NX118, fig. 10) ordinarily giving a relatively flat curve at $\frac{1}{4}$ L_h, regular sera (G.G.C. 3768, G.W.P. 160)

appear avid, irregular sera (S 601, S 602) non avid, when tested at 1 L_h and $\frac{1}{4}$ L_h . It appears then that if the toxin antitoxin complex with regular sera is relatively unstable, all sera tested against the toxin will appear non avid, if the toxin antitoxin complex is stable with regular sera, non avidity is shown by irregular sera only. Shortage of animals has made a thorough in-vivo investigation of these systems impracticable.

Titration of complex toxins

If a serum containing both α and θ antibody is titrated hæmolytically against a mixture of the two toxins, the answer obtained will depend on whether the ratio $\frac{\alpha \text{ units}}{\theta \text{ units}}$ exceeds or is less than the ratio of the combining powers of the corresponding toxins in the test dose. Thus if $\frac{\alpha \text{ units}}{\theta \text{ units}}$ is greater than $\frac{\alpha \text{ combining power}}{\theta \text{ combining power}}$, the θ value will be obtained, and vice versa. Therefore if by any means we can so increase the estimated α combining power of an $\alpha + \theta$ toxin that the ratio $\frac{\alpha \text{ combining power}}{\theta \text{ combining power}}$ is greater than $\frac{\alpha \text{ units}}{\theta \text{ units}}$ instead of less, we should obtain the α value instead of the θ value of the serum. Moreover, it is clear that if the ratio $\frac{\alpha \text{ units}}{\theta \text{ units}}$ of two sera is greatly different, we might by adding CaCl_2 obtain the α value for one serum and the θ value for the other, whereas if no CaCl_2 were added the θ value would be obtained for both. Consequently even if sera are tested against the same standard serum, different values may be obtained under different conditions. Table XIII shows the sort of result obtained. It is natural to

TABLE XIII

Serum values obtained by titrating mixed $\alpha + \theta$ sera against a mixed $\alpha + \theta$ toxin, using different diluents and the same standard serum

Serum	Real values		Diluent for toxin	Serum values
	α	θ		
A	0.5	10	B B S	9.1
			0.8 per cent CaCl_2 in B B S	3.7
B	1.0	10	B B S	10.0
			0.8 per cent CaCl_2 in B B S	6.3
C	2.0	10	B B S	10 (taken as standard)
			0.8 per cent CaCl_2 in B B S	10 (taken as standard)

With B B S as diluent, all three sera give the θ end point, with 0.8 per cent CaCl_2 in B B S, C gives the θ end point, while A and B give the α end point.

suppose that all except technical errors can be eliminated by a control titration under the same conditions of a standard serum against the toxin used in the test. What we have shown is that, if this method of testing is used, the ratio $\frac{\text{test serum}}{\text{standard serum}}$ may depend on the conditions themselves. It may be objected that no investigator is likely to dissolve his toxin or dilute his serum in CaCl_2 ; but some workers dissolve their toxin in broth, the calcium in which may be sufficient to make a considerable difference to the answer. The reverse procedure (that of reducing the α combining power of the toxin) is readily achieved by dilution in phosphate buffers or oxalate, with results which are easily appreciable.

No indicator for titrations of complex toxins or sera should be considered satisfactory which fails to indicate for which of the toxins and antitoxins the end-point is being determined; the fact that different answers may be obtained for a particular test toxin and serum by varying the conditions may occasionally be useful in demonstrating the complexity of the systems investigated.

Titration of α toxoids

Nagler (1941) has claimed that, if alum-precipitated toxoids are dissolved in sodium citrate to one-half the volume of original toxin and made up to volume with 1 per cent. CaCl_2 , the combining power of the toxoids can be estimated by haemolytic titrations of mixtures of toxin and toxoid against antitoxin. Our experience is that the estimated combining power of α toxoids depends on the amount of α toxin with which they are mixed; the lower the concentration of toxin the lower the estimated combining power of the toxoid. Mixtures of tetanus toxin and toxoid behave in a similar manner when tested *in vivo* against non-avid sera. Here

TABLE XIV

Effect of CaCl_2 concentration on combining power of alum-precipitated toxin redissolved in sodium citrate

Final concentration of CaCl_2 (per cent)	Combining power of 1 c.c. of dissolved A P toxin
0	0
0.0125	0
0.025	0
0.05	<0.2 α
0.1	0.25 α
0.2	0.16 α
0.4	0.51 α
0.8 *	0.62 α
1.6 *	<0.4 α (end-point unsatisfactory)

* Precipitation of calcium citrate.

the failure to detect all the toxoid present seems to be due to the fact that non-avid tetanus sera hardly combine with toxoid at all. We have also found that direct titration of alum-precipitated toxins dissolved in 2 per cent. sodium citrate gives values for the combining power dependent on the final concentration of CaCl_2 . Table XIV shows the estimated combining power of an alum-precipitated toxin derived from liquid toxin NN951 (1 c.c. \equiv 1.5 α units) dissolved to half the original volume in 2 per cent. sodium citrate and then diluted to the original volume with varying concentrations of CaCl_2 dissolved in B.B.S.

Routine in-vitro α testing

From our observation, rules can readily be drawn up to govern α testing.

1. The test dose should be as high as is economically practicable. We suggest testing at $\frac{1}{2}$ or 1 α unit.

2. Liquid toxins owing to their relatively high calcium content are preferable to dry toxins. The test dose should be re-determined at regular intervals to allow for fall in titre. The test toxin should preferably be antigenically pure α ; if such toxins are not produced by the strains available and significant amounts of θ toxin are present, they should be neutralised with appropriate amounts of θ antitoxin free from α antitoxin. As θ toxin does not affect L.V., this precaution is strictly necessary only in hæmolytic testing.

The hæmolytic and L.V. values of typical regular α antisera such as G.G.C. 3768 and G.W.P. 160, determined at the same test dose against the test toxin, should not differ by more than 10 per cent. Toxins giving differences greater than this should not be used, as with them it is not easy to determine degrees of avidity in sera.

There is naturally no certainty that toxins produced from all strains will have approximately the same calcium content. Determinations of calcium concentration should therefore be made, and if the concentration is *much* less than the equivalent of 0.05 per cent. CaCl_2 , it should be brought up to this level by the addition of CaCl_2 to the bulk toxin. Judging from our own experience this will seldom be necessary. Dilutions of liquid toxins should be made in B.B.S. containing 0.05 per cent. CaCl_2 and 1 per cent. Parke Davis peptone.

3. Dry toxins should be dissolved in 0.05 per cent. CaCl_2 + 1 per cent. Parke Davis peptone in B.B.S. of pH 8. They will then behave very much as liquid toxins do. Toxins so dissolved can be selected for testing by the method used for liquid toxins (*vide supra*).

4. The standard serum must not be irregular; when tested against a typical liquid toxin the hæmolytic and L.V. values at the same test dose should not differ by more than 10 per cent.

5. Owing to its high calcium content and relative insensitivity to changes in calcium concentration, L.V. is the most satisfactory general indicator. It also has the advantage of speed; several consecutive tests on the same serum can be done during the day. As high concentrations of serum tend to prevent aggregation of fat, L.V. is unsuitable for testing sera whose value is less than about 10 α units. If L.V. is made in a constant manner its calcium content is fairly uniform.

6. Determination of α values of antitoxins from 10 to $\frac{1}{100}$ α units is best done by hæmolytic testing on serum—never on plasma. As no great accuracy is usually necessary, no notice need be taken of the increase in estimated combining power of the toxin due to the relatively high concentration of serum.

Method. From 10 to 1 α unit values can be determined as for values greater than 10 α (see below), except that red cells must be used as the indicator.

For values below 1 α unit dilute the toxin in 0.05 per cent. CaCl_2 in B.B.S. to give convenient sub-multiples of the L_h —e.g. $\frac{1}{10}$, $\frac{1}{20}$, $\frac{1}{40}$, $\frac{1}{80}$, $\frac{1}{160}$, $\frac{1}{320}$, $\frac{1}{640}$ —in the test volume. To this volume of each dilution add 0.5 c.c. of the undiluted test serum, or less if the serum is markedly hæmolytic to sheep red cells. Mix by inversion over squares of non-absorbent paper. Put up a control in which 0.5 c.c. of normal horse serum ($< \frac{1}{100}$ α) is added to the test volume at each level. Mix. Allow both to stand for $\frac{1}{2}$ –1 hour, add 0.5 c.c. of 6 per cent. sheep red cells to each tube, incubate for 1 hour at 37° C. in the water-bath, allow to cool and read next morning. The error of one test is about 50 per cent.

7. If the value of the serum is above 10 units, preliminary tests should be done using L.V. as indicator; in the final determinations parallel tests should be carried out at the same time with red cells and L.V. as indicators. If there is a difference greater than 10 per cent. between the hæmolytic and L.V. values of the serum determined against the same test dose of a typical liquid toxin, the serum should be regarded as irregular and probably non-avid. A standard serum should always be titrated for comparison.

Method. To the test dose of toxin add volumes of serum suitably diluted in B.B.S. and differing by convenient amounts—in preliminary tests 50 per cent., in final tests 10 per cent. Make up to constant volume with B.B.S. Mix by inversion over non-absorbent paper, allow to stand half-an-hour and add 0.5 c.c. of indicator (6 per cent. sheep red cells, or L.V. prepared by the method of Macfarlane *et al.*). Red cells need not be mixed in, L.V. must be. Incubate for 1 hour at 37° C. in the water-bath, allow to cool and read next morning. For L.V. testing the crude end-point should be used as the indicating tube; in hæmolytic testing any convenient positive end-point is adequate; we have used 20 per cent. hæmolysis. Rough results can usually be obtained one hour after removal from the water-bath. The error of one test is about 10 per cent. at 10 per cent. differences, 25 per cent. at 50 per cent. differences.

8. All glassware used should be thoroughly washed at least twice in distilled water and dried before use. If new glassware is

used without washing for hemolytic testing, the results are often extremely irregular

Since in most laboratories a standard method of toxin production yielding a fairly uniform toxin with a calcium concentration not varying more than 50 per cent round the mean is sooner or later adopted, little difficulty is likely to arise in the comparison of α antisera with standards by *in vitro* methods, so long as a uniform technique is adopted. Small variations in calcium concentration in toxins are of little importance except in the titration of irregular sera. In these laboratories for instance thousands of sera have been tested by *in vitro* methods, and save in sera now known to be irregular, the methods have shown excellent agreement with one another and with lethal tests in mice. When, however, comparisons are attempted between toxins grown on media of widely varying composition, the results are unlikely to be reliable unless the concentration of calcium in the toxin is taken into consideration.

DISCUSSION

Apart from their importance in α toxin antitoxin testing, the experiments here described throw some light on testing for toxins and antitoxins in general. Considering α testing first, it is evident that a number of apparently simple assumptions will have to be reviewed

1 That the ratios between the L_+ , L_h and L_n , and those between the MHD , MND and MLD of an antigenically pure toxin are constant for all samples of that toxin. Since by varying the calcium content of an α toxin we can reduce its MHD and its minimal opalescing dose for serum without altering the MND and MLD , the ratios between these values can be made to vary. Similarly the L_h can be made to vary without affecting the L_+ or L_n .

2 That inspection of the ratios $\frac{MLD}{MHD}$, $\frac{L_n}{L_+}$ and $\frac{L_n}{L_h}$ for a particular type A toxin will give information on the purity or even the antigenic composition of the toxin. Thus Ipsen and Davoli (1939) state that pure α toxins have the characteristics

$$\frac{MLD}{MHD} < 1, \frac{L_n}{L_+} = 1.15, \frac{L_n}{L_h} = 0.8-1,$$

whereas θ toxins containing a trace of α give

$$\frac{MLD}{MHD} > 100, \frac{L_n}{L_+} > 1.5, \frac{L_n}{L_h} > 1$$

Under different conditions our toxin NX121 gives the values shown in table XV. Owing to the different levels at which the titrations have been done, concurrence with the values obtained

by Ipsen *et al.* could hardly be expected; we lay stress only on the fact that we can make the values vary.

TABLE XV

Minimal indicating and test doses of toxin NX121 at different calcium concentrations

Molar concentration of calcium in toxin	M.H.D. (mg.)	M.L.D. (mg.)	M.L.D. M.H.D.	L. ₊ (mg.)	L _n /10 (mg.)	L _h /4 (mg.)	L _n /10 L. ₊	L _n /10 L _h /4
3.7×10^{-5}	0.01	0.03	3	1.7	0.12	2.5	0.07	0.018
4.5×10^{-5}	0.0006	0.03	50	1.7	0.14	0.87	0.08	0.160

As the L_h is never found to equal $4 \times L_h/4$ if the toxin is diluted in B.B.S., the observed values are given and not L_h or L_n values derived from them by multiplication.

3. That the ratio $\frac{L_h}{M.H.D.}$ is some indication of the amount of toxoid present—the lower the value the more toxoid. Table IX gives values for this ratio for toxin NN540 under different conditions and shows the unreliability of this type of argument for α toxin. Nor can it be claimed that our experiments have been done under conditions far removed from those occurring in practice, for the calcium content of α toxins varies very widely, owing to the varying degree to which the CaCO₃ added to the medium is dissolved by the acid produced during growth. Nineteen liquid toxins examined by us had a calcium content varying between 3 and 6×10^{-3} M. Dry toxins contain very little calcium per L_h; NX121 had only 3.7×10^{-5} M. when dissolved in B.B.S. so that 1 c.c. \equiv 1 α hæmolytically. Differences may therefore be expected between dry and liquid toxins in hæmolytic testing. The use of L.V. as indicator will tend to give higher values for the combining power of α toxins than hæmolytic testing: the discrepancy will be greater for dry than for liquid toxin. The higher the concentration of calcium in the toxin, the greater will be the difference between the M.L.D., the M.N.D. and the M.H.D., and between L₊, L_n and L_h (table XVI); the methods are not accurate enough to show much difference when the calcium values lie close together, especially at the low test doses which are essential in skin testing, but the difference between the dry and liquid toxins is obvious.

It is well known that sub-multiples of the test dose do not necessarily measure equivalent sub-multiples of combining power. If the serum is avid, sub-multiples of the test dose, owing to the reduction in the number of indicating doses, will require relatively less serum for neutralisation; if it is non-avid, they may require relatively more. But we are not aware of any toxin-antitoxin system where variation of in-vitro conditions at constant volume

will alter the apparent combining power. The implications are obvious enough and are particularly important when physical methods of examining α toxin are under consideration. Let us

TABLE XVI

Test doses of various α toxins at $\frac{1}{10}$ α unit

Toxin	Condition	Molar concentration of calcium	L_{+} (c c)	L_{n} (c c)	L_{h} (c c)
NN937	Liquid	2.5×10^{-4}	1.5	1.35	1.2
NN953	"	3.1×10^{-4}	0.95	0.75	0.65
NN957	"	3.1×10^{-4}	0.85	0.8	0.6
NN958	"	4.3×10^{-4}	1.2	1.0	0.7
NN903	"	3.6×10^{-4}	2.0	1.8	1.5
NN924	"	4.6×10^{-4}	0.9	0.85	0.6
NX111	Dry, dissolved in BBS	1.2×10^{-4}	0.85	0.8	0.8
NX118		9×10^{-5}	0.53	0.5	0.5
NX119		7×10^{-5}	0.53	0.5	0.5

suppose for instance that ultracentrifugation, diffusion, or ultrafiltration is being used under optimal conditions to determine the particle diameter of α toxin, and that the toxin has been calibrated by diluting it in BBS and constructing a graph from which the real fraction of the original toxin present can be estimated from its apparent combining power.

1 *Ultracentrifugation* The toxin particles will be centrifuged out more rapidly than calcium ions, examination of the combining power of the supernatant will therefore indicate more toxin than is really present (considered as a fraction of the total). The value for the particle size will therefore be too small.

2 *Diffusion* Calcium ions will diffuse more rapidly than toxin, the apparent combining power of the diffused toxin will be too great and the particle size deduced too small.

3 *Ultrafiltration* Calcium ions will ultrafilter more readily than toxin, more toxin will be determined in the filtrates than is really present and the filtration curves will be inaccurate in form. Moreover, since the calcium salts are readily washed through membranes which retain toxin, it is unjustifiable to assume that washings from such membranes made up in calcium free diluents contain no α toxin because they do not haemolyse red cells.

Since the discrepancy varies with different toxins and sera, the only way out of the difficulties is either to use a method which does not involve the use of an indicator (such as flocculation) or to calibrate the toxin and serum which are to be used over a wide range of calcium concentrations and use these curves to correct the values found in experiments. Some workers (*eg* Reed *et al*, 1939) add magnesium salts to their media, these must also be taken into consideration.

Notwithstanding the fact that all our toxins have been derived under practically uniform conditions from the same strain (S.R. 12), they show marked differences in their behaviour towards α antisera. Inspection of calcium avidity curves shows clearly that the stability of the toxin-antitoxin complex varies with the toxins as well as with the sera; the value m gives a rough idea of the stability of the complex—the lower it is the more stable the complex. Barr and Glenny (unpublished observations) have shown that the dilution ratio for any particular diphtheria antitoxin may vary with the toxin used for the test. They refer to the “affinity” of toxin and toxoid for serum as compared with the “avidity” of sera for toxins and toxoids. They found that different batches of diphtheria toxoid varied in affinity for antitoxin as compared with a standard toxin. Similarly Glenny and Stevens (unpublished) found that if sera RX 3102 and F.C. 3416 were tested against tetanus toxins 165 and 179 with a constant standard serum the apparent value for RX 3102 was higher with toxin 165 than with 179, while with F.C. 3416 the reverse was the case. In other words RX 3102 “fitted” 165 better than 179; F.C. 3416 “fitted” 179 better than 165.

Calcium avidity curves afford a method of determining goodness of fit between α toxins and α antisera; it remains to be seen whether toxins giving a good fit with antisera have higher immunising value than those whose fit is poor.

In estimating toxin or antitoxin it is usual to mix constant amounts of one component with varying amounts of the other and determine the “neutral” point. Two types of method are available—those involving the use of indicators and the flocculation method. When a mixture of toxin and antitoxin affects an indicator it is natural to assume that at least one minimal indicating dose of toxin was present in the mixture before the indicator was added. In other words the indicator (red cells, lecithovitellin, serum, animal body) does not interfere with the reaction system but merely indicates any amount of toxin greater than one minimal indicating dose. If the argument on p. 345 based on the law of mass action applies to the present case, it is clear that this may not be true and that the addition of an indicator may so upset the equilibrium of the system that toxin is set free by dissociation of the toxin-antitoxin complex. How much is set free will depend on the stability of that complex, and on this also will depend the amount of antitoxin which has to be added to repress its dissociation. The result of all this is that the neutral mixture in an indicating system will ordinarily contain more antitoxin than strict molecular neutrality would demand.

Dilution of “neutral” toxin-antitoxin mixtures will also give results dependent on the stability of the complex. Dilution of an

equilibrium mixture by two will reduce $[T]$ and $[A]$ to one-half, as well as $[TA]$. The equilibrium ratio will therefore be reduced to $K/2$ and TA must dissociate to restore the old value K . The degree of dissociation will depend on the stability of the complex, and on this also will depend the effect of adding the indicator. The final result will depend on whether the extra toxin set free by dilution and the addition of the indicator is sufficient to offset the reduction in total indicating doses due to dilution.

The interest of the α toxin-antitoxin reaction lies in the fact that we can at will favour the in-vitro indicator reaction (e.g. calcium ions + toxin + red cells \rightarrow hæmolysis) and thereby greatly alter the toxin-antitoxin equilibrium, with correspondingly marked effects on the estimated combining power of the toxin.

In a flocculation test, the tube flocculating first is regarded as neutral; and since no indicator is present to compete with the antitoxin for toxin, it might be hoped that the neutral point as estimated by flocculation would be a more exact determination of molecular neutrality than could be obtained by any method involving indicators. Certainly α toxin-antitoxin flocculation is unaffected by the concentration of calcium present, but unfortunately the answers obtained for dry toxins are usually much higher than those obtained by indicator methods, which, unless large amounts of toxoid are present, is the reverse of what would be expected.

SUMMARY

1. The estimated value of the minimal indicating dose of α toxin against red cells, human serum and lecithovitellin depends on the concentration of ionised calcium present. Increase in the calcium or magnesium concentration leads (up to a point) to a decrease in the minimal indicating dose.

2. The minimal lethal dose and the minimal necrotising dose are independent of the calcium concentration, presumably owing to the free diffusibility of calcium ions.

3. The estimated combining power of α toxin is increased by increase in concentration of calcium or magnesium; the relationship is logarithmic: $\log. \text{ combining power} = m (\log. \text{ molar concentration of calcium or magnesium}) + \text{a constant}$. The value of m varies greatly for different toxins and sera. An explanation of this is suggested on the basis of the law of mass action.

4. α Toxins differ qualitatively among themselves in the stability of the compounds they form with α antisera; these differences can be demonstrated and measured by "calcium avidity curves".

5. The combining power as determined by intravenous inoculation of toxin-antitoxin mixtures into mice or intracutaneous

injection into guinea-pigs is unaffected by changes in calcium concentration.

6. The neutral point in flocculation tests of α toxin-antitoxin mixtures is unaffected by changes in calcium concentration.

7. On these findings an explanation is suggested for the differences in serum values obtained by titration against various dry and liquid toxins, using red cells and lecithovitellin as indicators.

8. The effect of these observations on the titration of α toxin and antitoxin and their general bearing on toxin-antitoxin testing is discussed.

We wish to express our gratitude to Mr A. T. Glenny for constant interest and innumerable suggestions as well as for most generous supplies of sera, to Miss H. E. Ross and Mr A. Thomson for large supplies of both dry and liquid toxins, to Miss I. M. Tonkin for the substance of table XVI, to Dr C. G. Pope for the substance of table VIII and to Miss M. Barr, Miss D. M. Marvell and Dr R. G. Macfarlane for much useful information.

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EXPERIMENTS ON DRYING AND ON FREEZING BACTERIOPHAGE

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COMPARATIVELY little work has been done on the desiccation of bacteriophage Knorr and Ruf (1934 35) dried small quantities on coverslips and on mica at room temperature without a vacuum for varying times up to 2 hours They found that the greatest drop in titre occurred when the phage became partially dry Thus the titre of a *Bact coli* phage dropped from 76 per cent of the original titre after 5 minutes' drying to 6.7 per cent after 10 minutes Although this phage and a *Bact typhosum* phage were largely destroyed by drying, a *Bact dysenteriae* Flexner phage was unaffected

The aims of my experiments were to ascertain by quantitative measurements (1) whether different phages could be classified according to their sensitivity to destruction by desiccation, (2) whether, by drying, different types could be isolated from a mixed phage and (3) suitable media for the preservation of the phage during drying Experiments on freezing undried and partially dried phage were also made

TECHNIQUE

Stock bacteriophages were propagated periodically on their homologous cultures in the following medium, suggested by Dr A. L. Robinson

NaCl	0.50 g
MgSO ₄	0.02 "
K ₂ HPO ₄	0.10 "
Sodium citrate	0.20 "
(NH ₄) ₂ SO ₄	0.10 "
Glucose	0.10 "
Peptone ("Bacto" brand)	0.01 "
Distilled water	100 c.c.

The pH was adjusted to 7.8 After 24 hours' incubation at 37° C the phages were filtered through L 5 or L 3 Chamberland filters and stored in the ice chest

The phage was diluted in Robinson's medium to the required titre and one standard drop was dried by the following procedure With a Dreyer standard dropping pipette, one drop of the suspension was put into each of a series of sterile test tubes of a uniform interior diameter of 8 mm At the same time one drop was put into a sterile dwarf test tube containing 9 standard drops of Robinson's medium This control sample was kept in the cold store until the time came to test it together with the dried drops

The tubes containing the samples to be dried were put in a glass container in a desiccator containing a small basin of phosphorus pentoxide and fitted

with a manometer. The wool stoppers were taken out of the tubes, and the desiccator evacuated with a Cenco Hyvac pump for 30 minutes. The samples froze within 4 minutes under these conditions and remained so until "dry". After this the tubes were left *in vacuo* in the desiccator at room temperature for a given time. When the tubes were taken out of the desiccator 10 drops of Robinson's medium were immediately put into each—allowing for the evaporation of the dried drop, this brought the samples to one-tenth of their original titre. The samples were then further diluted in a series of dwarf test tubes using Robinson's medium as the diluent. A fresh Dreyer pipette was used for making each dilution; the pipettes were sterilised by boiling in distilled water and dried by a suction pump with acetone and flaming. Ten-fold dilutions were usually made and each dilution was thoroughly mixed by sucking up and down in the pipette.

Petri dishes, each containing 10 c.c. of 1.8 per cent. nutrient agar, were flooded with a 24-hour growth of the appropriate microbe in Robinson's medium; the excess was pipetted off and the inoculated plate dried for one hour at 37° C. with a sheet of filter paper in place of the lid. The plate was then placed on a paper marked with seven circles which could be seen through the agar; by this means seven standard drops could be put symmetrically on each plate. With experience of a phage it was possible to foretell fairly well in which of the 10-fold dilutions of the control and of the dried samples 10-100 plaques in a standard drop would be found. The plates were then incubated at 37° C. for 18-24 hours and the plaques in each inoculated area counted. The counting was facilitated by ruling lines in ink on the bottom of the plate. By holding the plate below a lamp against a dark ground the plaques could be easily counted with a hand lens.

Calculation of results: the "standard curve" and the "index of destruction"

The "standard curve" of Dreyer and Campbell-Renton (1933) was devised because, when a series of dilutions of phage is plated, the number of plaques formed is not exactly proportional to the concentration of the phage. The ratio between bacteriophage concentration and the number of plaques formed was represented graphically by a standard curve based on actual experiments. This standard curve constitutes a basis for the quantitative estimation of the concentration of any bacteriophage. Yen (1934-35) found that this standard curve fitted his observations with a *Bact. dysenteriae* Shiga phage.

A like phenomenon is seen with vegetable viruses. Bawden and Prio (1938, p. 75) in their experiments with potato virus "X" found that "The decrease in numbers of local lesions accompanying dilution is not directly proportional to the dilution, but more are obtained at high dilutions than would be expected". With the tobacco mosaic virus the deviation from the straight line was even more noticeable.

The viability of the phage in the control and in the dried drops was determined as follows. The "phage units" which corresponded to the number of plaques counted in each area were read off from the standard curve. The fraction expressing the dilution, multiplied by 10^5 and divided by the number of phage units corresponding to that dilution gives a figure which is called the K of the sample. The K of each dried sample divided by the K of an undried control on the same plate gives a ratio which I have called the "Index of destruction", or for brevity the "I.D." ($\frac{100}{\text{I.D.}} = \text{percentage of phage surviving}$).

Table I shows an experiment with the phages of staphylococcus "Pasteur" and *Bact paratyphosum B* ("D M large") to illustrate the method of calculation. In this experiment equal volumes of the two phages were mixed together before desiccation and drops of the control and of the dried mixture were put on plates inoculated with each microbe. It will be seen that, under identical conditions, the staphylococcus phage is 188 times more sensitive to the destructive effects of drying than the "D M large" phage.

Variability of experimental results

When the plaques in an area numbered about 10-100 the variation in the results obtained from plating drops in duplicate or triplicate was not great. In 31 experiments where the number of plaques lay between 5 and 70 the average standard deviation from the mean among duplicate drops was 10.7 per cent*. In another experiment when seven drops of an undried phage in a dilution of 1-100,000 were plated, the average count was 84 plaques per drop and the standard deviation 18.8 per cent. Seven drops of a dried phage in a dilution of 1-200 gave an average count of 103 plaques and a standard deviation of 9.3 per cent.

In order to see whether six dried samples gave an average I.D. comparable with that of a larger number of samples, 27 sample drops of *Bact paratyphosum B* ("G 1") phage were dried for three days and diluted to 1-100. Drops from 6 of these samples were plated separately and a mixture was then made of the 27 samples, taking two drops from each. This mixture was plated in triplicate. The average I.D. for the 6 samples plated separately was 358 and for the 27 mixed samples 368, which showed good agreement.

Although individual dried samples in a series of six may show considerable differences in I.D., the average of sets of experiments of 4 to 6 samples made at different times shows very little variation. The average I.D. for "G 1" phage in 15 experiments was 395 with a standard deviation of 35.4 per cent. Nine experiments with staphylococcus "Pasteur" phage had an average I.D. of 333 with a standard deviation of 44.7 per cent. Seven experiments with "D M large" phage had an average I.D. of 13 with a standard deviation of 21.2 per cent. The "G 1" phage and the staphylococcus "Pasteur" phage show no significant difference in their sensitivity to drying, but the "D M large" phage differs from both to an extent outside the range of any experimental variation.

* The standard deviations are expressed as percentages of the mean

with a manometer. The wool stoppers were taken out of the tubes, and the desiccator evacuated with a Cenco Hyvac pump for 30 minutes. The samples froze within 4 minutes under these conditions and remained so until "dry". After this the tubes were left *in vacuo* in the desiccator at room temperature for a given time. When the tubes were taken out of the desiccator 10 drops of Robinson's medium were immediately put into each—allowing for the evaporation of the dried drop, this brought the samples to one-tenth of their original titre. The samples were then further diluted in a series of dwarf test tubes using Robinson's medium as the diluent. A fresh Dreyer pipette was used for making each dilution; the pipettes were sterilised by boiling in distilled water and dried by a suction pump with acetone and flaming. Ten-fold dilutions were usually made and each dilution was thoroughly mixed by sucking up and down in the pipette.

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upon the spinal canal. Other tumour masses were present in the iliac bones, sternum and nearly every rib. The lungs were congested and oedematous and there were areas of confluent pneumonic consolidation in the left lower lobe. The heart weighed 350 g. and the normal relationships were preserved. The right and left kidneys were enlarged, weighing 170 and 225 g. respectively. There was marked pallor, especially of the cortex, and the organs had a firm but not rubbery feel. On section they were more resistant than normal and the cut edge remained sharp. The cortex was indistinct and showed no pattern; it was but slightly reduced in width. The capsule stripped easily, leaving a smooth and rather yellowish-grey surface marked only by a few prominent veins.

Microscopical examination. The tumour masses presented the histological structure of multiple myelomata. Their cells were of plasma cell type but their cytoplasm failed to stain specifically. The kidneys presented an unusual picture. The most marked change affected very many of the tubules, especially the distal convoluted and collecting tubules and to a lesser extent the ascending limbs of the loops of Henle. An unorganised but sometimes laminated material blocked the lumen and compressed the lining cells (figs. 1 and 2). Debris of epithelial cell origin contributed nothing to the formation of these casts. Only some tubules were much dilated by the casts, which very rarely approached the width of a glomerulus. In relation to these there was often regeneration of epithelial cells, and though these were within the tubules they often resembled the giant cells of a foreign-body reaction. True giant cells lying in relationship to the reticulum were very rare. The characteristic appearance of the lining cells of the tubules at different levels was somewhat altered. This was partly due to post-mortem change. There was also some epithelial cell regeneration and nuclei were sometimes slightly increased, even in segments devoid of casts. More often the lumen of the tubules was collapsed and many tubules were atrophied. Regressive changes were occurring without the presence of significant fatty or hyaline droplets, but aniline blue staining showed that there was some thickening of the basement membrane of these tubules. The changes were diffuse. There was very little inflammatory cell infiltration. The amount of reticulum contributed by the collapse and condensation of the intertubular framework following the atrophy and removal of the tubular epithelium was considerable in some areas (fig. 1). In this, strands of collagen were in process of formation and the interstitial tissue throughout the organ was greatly increased. The glomerular changes were remarkably uniform though varying slightly in degree. Less than 4 per cent. of the glomeruli were effete or hyalinised. There was some slight thickening of the capsular basement membranes and of the pericapsular fibrous tissue. Where marked,

this was associated with some atrophy of the glomerular tuft. Occasionally the capsular lining cells were very slightly prominent but there was no crescent formation. The afferent arterioles were often slightly dilated and the glomeruli appeared relatively avascular. Thickened strands, staining reddish brown with van Gieson and blue like the basement membrane with aniline blue, altered the pattern of the glomeruli, giving them a more lobular appearance (fig 2). This material occupied a relatively central position in the glomerulus and merged with the glomerular basement membrane, which thus appeared greatly thickened, though only in parts. The basement membrane of the peripheral loops showed no distinctive thickening or fibrillation. In the thickened strands a large amount of material silvered like reticulum (fig 1). It was often continuous with the reticulum at the hilum. The glomerular lesion resembled the primary degeneration of the intercapillary connective tissue as described by Kimmelstiel (1935). There was the same pattern and apparent relationship of the axial strands to the lobular capillaries. There were no hyaline intracapillary fibrillæ as in glomerulonephritis (McGregor, 1929b) and stains for amyloid were negative. There were no hyaline changes in the arterioles and the arteries showed very little intimal proliferation.

Apart from oedema, congestion and some inflammatory changes in the left lung the other organs presented no abnormality. Special attention was directed to the blood vessels.

Summary In this case casts, not formed by epithelial debris, occupied the lumen of many of the renal tubules and were associated with regressive changes and atrophy of a great part of the tubular epithelium. Removal of many effete tubules was associated with collapse of the reticulum, extensive fibrous tissue reaction and distortion of the renal architecture.

Case 2 (A 2511)

Clinical history A housewife, aged 43 years, gave a 6 months' history of sharp darting pains in the legs, later becoming a dull ache. There was no wasting and no oedema. The hæmoglobin was 46 per cent and the blood pressure averaged 144/82 mm Hg. X-ray examination showed rarefied areas in the ribs and vertebræ. Bence Jones protein and large amounts of albumin were passed in the urine but renal function was not studied. Twenty days before death complete paralysis of the legs developed.

Post mortem Myelomatosis (plasma cell type) with involvement of the vertebræ and ribs. Invasion of the epidural space, spinal paraplegia. Interstitial pancreatitis. Fat necrosis in mesentery. Diffuse fatty changes in liver. Casts in renal tubules. (Macroscopically the kidneys appeared normal.)

Microscopical examination The renal changes were slight and rather focal in character. Only a few tubules, chiefly the distal

convoluted and collecting tubules, showed casts and these were small. Around the casts, but only to an insignificant degree elsewhere, there was proliferation of the tubular epithelium. The cells of the proximal convoluted tubules showed necrolytic changes, but the complete absence of reaction or inflammatory cell infiltration indicated that this was a post-mortem development. Dilatation of the proximal tubules and capsular spaces was absent. Occasional giant cells represented tubules which had contained casts and, with a few small focal scars, served to indicate the duration of the condition. Very few glomeruli were hyalinised but a few showed some thickening of the capsular basement membrane. Reticulum in the glomerular tufts was limited to the immediate neighbourhood of the hilum.

Summary. In this case the lesions were relatively slight and the general architecture was not distorted. Comparatively few tubules were involved, though the lesions were of some duration.

Case 3 (A 2866)

Clinical history. A labourer, aged 59 years, complained of marked increase in long-standing epigastric discomfort and of recent loss of appetite and extreme weakness and tiredness. He had vomited frequently during the preceding 6 weeks and had lost weight. Examination revealed a microcytic anæmia with hæmoglobin 50 per cent., blood pressure 120/70, and no radiological evidence of any intestinal lesion. The blood urea was 201 mg. per cent. and rose during the 17 days before death to 330 mg. per cent., when the blood creatinine was 9.5 mg. per cent. A urea range test gave fixed specific gravities of 1012 and 1010. Three days before death the urine was found to contain Bence-Jones protein as well as considerable amounts of albumin. X-ray examination of the skull, thorax, lumbar region and pelvis failed to reveal any areas of rarefaction.

Post-mortem. Very numerous tumour nodules were found in relation to ribs, sternum and vertebræ above the level of the 3rd thoracic. They projected chiefly into the thoracic cage and there was remarkably little destruction of bone. The pelvic bones and skull were normal. There was no oedema and the serous cavities contained no excess of fluid. The heart showed no hypertrophy. The kidneys (170 and 210 g.) were pale and their architecture was indistinct, while the cortical zones were narrowed. Their capsules stripped readily, leaving smooth surfaces free from depressions. The organs were firm but lacked the solid feel of the kidneys of case 1.

Microscopical examination. The tumour tissue did not react to the Unna-Pappenheim stain and did not give the peroxidase reaction. Morphologically the cells were mostly of plasma-cell type. In the kidneys the casts, especially in the cortical tubules, were larger than in the previous cases (fig. 3). They sometimes approached a diameter twice that of a glomerular tuft. They were usually

RENAL TUBULAR OBSTRUCTION

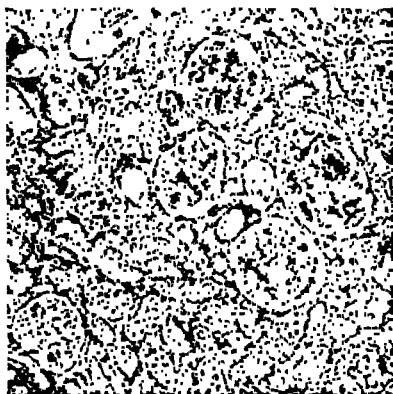


FIG. 1.—A 2559, case 1, multiple myeloma. Surviving and atrophic tubules, some containing casts, separated by increased reticulum and interstitial tissue. Silvering strands are prominent in the glomeruli. Foot's silver for reticulum and H. and E. $\times 90$.

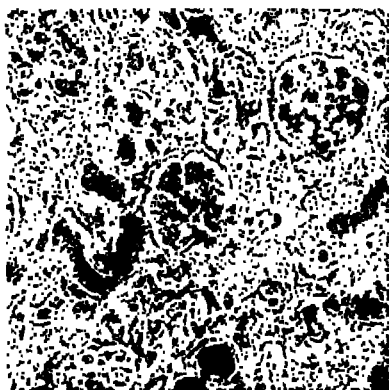


FIG. 2.—A 2559, case 1, multiple myeloma. Casts in renal tubules, increase of interstitial tissue and coarse lobulating strands in glomeruli. Mallory-Heidenham. $\times 90$.

laminated and gave several different coloured zones with many of the stains used. Material staining like fibrin was often present. The tubular epithelium in relation to the casts was flattened and often there was considerable epithelial proliferation. Nuclear aggregates resembling giant cells often lay in relation to the casts and almost always were definitely inside the tubules (fig. 4). Portions of tubules without casts in their lumen were often undergoing atrophy: epithelial basement membranes were slightly thickened and it was usually possible to distinguish them clearly from the reticulum. Proximal convoluted tubules survived in some areas and were well preserved: sometimes they were very slightly dilated. Fatty or hyaline degeneration was nowhere prominent. The reticulum appeared considerably increased, but some of this was due to collapse of the reticular sponge following tubular atrophy. Proliferation of fibroblasts and increase of collagenous connective tissue were somewhat less prominent than in case 1 and were best seen between the tubules in the medulla. A feature of this case was the presence of large numbers of inflammatory cells, including polymorphonuclear leucocytes. Some lay in tubules often in relation to somewhat disintegrating casts. More often they infiltrated between the remains of tubules. They were not present in the glomeruli. There was no infiltration of the sub-epithelial tissue of the pelvis. Only a few glomeruli were hyalinised. There was no dilatation of capsular spaces, though a few contained a little albuminous material. The capsular basement membrane and pericapsular tissue were often slightly prominent. There was a little thickening of the more central part of the tuft, but no thickening or fibrillation of the glomerular basement membrane and no dilatation or fixed patency of the capillary loops. Arteriolar and arterial vascular changes were absent in the kidney as in the other organs of the body.

Summary. Renal failure developed in a case where the formation and growth of large laminated casts in the tubules were associated with active tubular destruction. Associated with this there was marked inflammatory cell infiltration but less fibrous tissue reaction than in the previous cases.

Case 4 (A 2984)

Clinical history. A man, 64 years of age, gave a 3-months' history of lumbago. Radiographs at first negative later showed multiple areas of rarefaction in the pelvis and lumbar vertebrae. In hospital just before death the urine was found to contain albumin and Bence-Jones protein. The blood urea was 189 mg. per cent. on the day before death.

Post-mortem. The body was well developed and there was no wasting or oedema. Only a small lumbar incision was permitted. The kidneys and spleen and portions of the liver, suprarenals,

pancreas and tumour tissue were removed. The kidneys were slightly enlarged but of a normal consistency and colour. The cortex was not reduced and the cut surfaces showed a fairly normal pattern.

Microscopical examination. The tumour nodules were myelomata. The relatively anaplastic cells sometimes resembled the plasma cell type. The changes in the kidneys most closely resembled those in case 3 but were less marked. Casts were most frequent in the medulla and in some fields in the cortex no obstructed tubules were evident. In a relatively large number of tubules the lining cells were normal. In other small areas, evidently related to obstruction, tubular epithelium was being removed and there was considerable inflammatory cell infiltration of the stroma. Fibrous tissue reaction was very slight. Regeneration of epithelial cells was confined to the region of the casts and to some of the medullary collecting tubules. The number of hyalinised glomeruli and the vascular changes in the kidneys and other available organs make it difficult to exclude a mild degree of arteriolosclerosis, not enough of itself to cause any renal insufficiency.

Summary. In this case, casts, while often obstructing the lumen of the distal tubules, did not aggregate to fill the renal tubules so completely as in cases 1 and 3. There was, however, sufficient blockage of nephrons to produce renal failure.

Discussion

The dominant feature in all four cases is tubular blockage. There are also prominent changes resulting from the disintegration and absorption of those tubules whose lumen is blocked at some level. Changes in the epithelium of other tubules, which may still be functioning, are extremely slight. They are not comparable to those of lipoid nephrosis. In three cases (1, 3 and 4) the lesions are sufficient to have caused renal failure, though in the first case this was not proven clinically. In the first case the lesion is a slowly progressive one, the loss of parenchymatous tissue is not attended by inflammatory cell infiltration and nephrons are being gradually replaced by interstitial tissue. The kidneys feel firm and appear unusually pale. In case 2 lesions are confined to a few nephrons only. In cases 3 and 4, especially the first, the removal of tubular epithelium is associated with less fibrous tissue reaction but more inflammatory cell infiltration and the kidneys are somewhat less firm. Many of the casts have also been able to grow to a greater size. In all cases tubules are only dilated significantly at the actual site of deposition of a cast.

The nature of the casts in the tubules cannot now be determined, but they are not formed from degenerated cells of the tubular epithelium. They occur in those parts of the nephron where the

RENAL TUBULAR OBSTRUCTION



FIG. 3.—A 2866, case 3, multiple myeloma. Large casts in cortex dilating the renal tubules. Increase of fibrous tissue and inflammatory cells. H. and E. $\times 80$

FIG. 4.—A 2866, case 3, multiple myeloma. Casts in tubules in medulla; epithelial cells around one cast simulate a giant cell. Increase of fibroblasts and inflammatory cells. H. and E. $\times 90$.

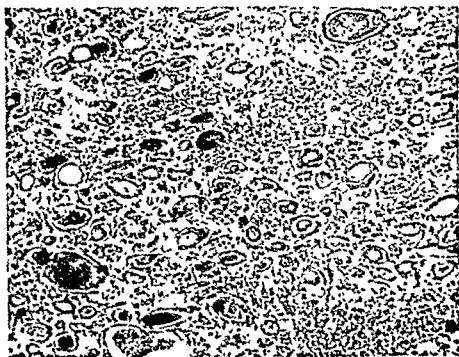


FIG. 5 —A 3005, case 6, crush anuria. Tubules in medulla showing casts. These are best marked in the collecting tubules; in any one plane of the section only a few tubules appear to be blocked. H. and E. $\times 90$.

urine is being concentrated, or where the urine is most concentrated and might best deposit material from solution. Their often laminated structure, with different tinctorial zones, suggests that any one cast is not all laid down at the same time. They may grow either by deposition from urine still able to pass down the tubules alongside them in spite of some obstruction, or by continued secretion and reabsorption of urine in the blocked nephron proximal to the obstruction. The laminated structure seems to support the first possibility. Oliver and Luey (1934) describe casts formed of granular debris in chronic glomerulonephritis and their growth by a process of aggregation. Such casts and the occasional hyaline casts found on careful examination in the tubules of so many otherwise normal kidneys are quite insignificant as compared with those of myelomatosis. One case of extensive amyloid disease has been studied in which renal failure was associated with dilated tubules filled with casts and with extensive intertubular fibrous tissue replacement. Here, however, the renal lesion was further complicated by vascular changes. The casts failed to give the amyloid reaction and their nature must remain uncertain.

Changes in the glomeruli are very slight, though plasma proteins were escaping as well as Bence-Jones protein. The condition of the glomerular basement membrane can best be studied in the peripheral loops of a tuft; no true thickening or fibrillation was detected there. Bell has claimed that in some cases of myelomatosis these changes occur and are comparable to those described by him (Bell, 1929) in lipoid nephrosis. Glomerular changes are only marked in case 1 (figs. 1 and 2), where radiating strands containing reticulum emphasise the lobular pattern of the glomerulus. They can best be described as intercapillary degenerative lesions. They may be related to alterations in the glomerular circulation but cannot be regarded as specific. Ischæmic atrophy of the glomerulus with thickening of its capsule is usually much more marked in hydro-nephrosis, where the vascular disturbance must be greater. Primary arterial or arteriolar changes can be completely excluded in at least three of the four cases.

The presence of large numbers of mononuclear cells and especially of polymorphonuclear leucocytes in the kidneys of cases 3 and 4 is of interest. There was no evidence that this represented extra-medullary myelopoiesis, either from the appearance of the cells themselves or from changes in other organs, nor did the amount of bone marrow destruction suggest such a possibility. The absence of inflammatory changes in the renal pelvis excludes a coincident ascending pyelonephritis. The obstruction of the tubules might favour the development of a hæmatogenous infection. Considerable inflammatory cell infiltration may follow the tubular destruction of uncomplicated renal vascular lesions. Bohnenkamp (1922) has

emphasised the extensive myeloid infiltrations which may follow the tubular necrosis of corrosive sublimate poisoning. In case 1 the stromal reaction is almost entirely fibroblastic in character.

CRUSH ANURIA

Three cases of crush anuria have been carefully studied at autopsy. The findings in all three are so similar that they may be described together.

Clinical observations

The first case (case 5, A 3003) was that of a man of 41 years who lived for 6 days. There was an associated lumbar cord compression from fracture of the spine, a fracture of the fibula and multiple abrasions. The second (case 6, A 3005) was that of a man of 26 years who survived for 8 days, the third (case 7, A 3006) a woman of 38 years who died on the ninth day. All had been buried under debris for periods of 4-8 hours. A striking feature in all three was the absence of really serious shock. One (case 6) received 500 c.c. of plasma on the day after admission and was later given glucose-saline and sodium sulphate intravenously without effect. These patients passed a few ounces of urine each twenty-four hours but specimens were only available from the sixth day in the last two cases. These were neutral or slightly alkaline and of specific gravity 1010-1015. Repeated attempts to demonstrate muscle hæmoglobin either in the urine or in the blood serum (using a Beck reversion spectroscope) failed. The terminal blood urea readings were 420, 594 and 351 mg. per cent. respectively. In case 7 the creatinine rose to 9.8 and the creatine to 16.0 mg. per cent.

Post-mortem findings

In all cases an autopsy was carried out before rigor mortis had developed and in case 6 within 2 hours of death. Œdema was marked over a fairly wide area around the site of crushing but was never completely generalised and the serous cavities contained no significant excess of free fluid. The subcutaneous adipose tissue of the crushed limbs was very oedematous but showed no pigmentation or extravasation of blood. On section of the muscle sheaths the muscles bulged through and exuded fluid. Muscle changes were present in all cases and the appearance described as "fish flesh" was very marked in cases 6 and 7. It was very irregularly distributed in the crushed limbs.

The kidneys in all cases were enlarged and appeared swollen, the cut edges bulging over the capsules, which separated very readily, leaving a smooth surface. They were congested and the medullary striæ were prominent. The demarcation between cortex and medulla and the cortical pattern itself were less distinct than normal.

The other organs presented no features of special interest.

Microscopical examination

In the areas of damaged muscle there was complete disintegration of muscle cells and considerable reaction and proliferation, resulting in the appearance of many prominent nucleated cells. Undamaged muscles gave a brownish colour with the benzidine reaction of Lepehne (1919) for hæmoglobin and allied substances. Often this was not evident in even slightly damaged muscle fibres. Traces of free iron were demonstrable in the fibrous tissue septa of damaged muscles. Free iron was present in the spleen, especially in case 6, and to a lesser extent in the liver. Other microscopic findings outside the kidney do not merit special mention. In none of the cases was there evidence of coincident hypertensive or degenerative vascular disease.

Kidneys The architecture was uniform and little altered. The tubules preserved their normal pattern and were separated by a fine reticulum without interstitial fibrous tissue reaction or scarring. The changes were most marked in the collecting tubules of the medulla (fig 5). In these there were compact brownish casts behaving like hæmoglobin or allied material with the benzidine test of Lepehne. Loops of Henle and distal convoluted tubules were less often involved and the deposits were often much less dense. The casts were not laminated but sometimes appeared slightly granular, though no red cells could be distinguished in the tubules. Epithelial debris and even organised elements such as inflammatory cells contributed relatively little to the formation of casts, especially in the larger collecting tubules. Albuminous debris and eosinophilic material were present in the proximal tubules and even sometimes in the glomeruli, especially in case 6. This appeared to represent protein containing fluid, probably somewhat concentrated and coagulated behind the more solid obstructions. At any one sectional level only a proportion of the tubules were blocked by the compact brownish casts, but as these did not fill the whole length of any one tubule it is legitimate to assume that almost all tubules were blocked at some level. At all events the appearance in the more proximal parts of the nephrons was fairly uniform. In case 6, where sodium sulphate had been given intravenously, there was slight and fairly uniform dilatation of the more proximal tubules, in cases 5 and 7 the change was less marked.

The tubular epithelium in relation to the casts was sometimes flattened and there was epithelial proliferation in many of these areas. Elsewhere it showed little change, but there was slight epithelial regeneration in some of the distal convoluted tubules and in the ascending limbs of the loops of Henle. Fat and hyaline droplets were absent. In case 6 the brush border of the proximal convoluted tubules was perfectly preserved. Very occasionally

inflammatory cells had passed out between the tubules, often in relation to a few epithelial cells which were breaking down. Traces of a yellowish brown pigment were present in the cells of the tubules. It was not soluble in chloroform. No free iron was present in the kidney. The presence of an unidentified golden-yellow pigment, the absence of iron pigment in the kidney and its occurrence in the spleen and liver, but not the occurrence of casts in the renal tubules have been noted to follow the injection of muscle hæmoglobin into the dog (Newman and Whipple). Attempts to demonstrate the spectrum of myohæmoglobin by microspectroscopy of the perfused kidney of case 7 and in kidney extracts were unsuccessful.

Dilatation of the glomerular spaces was insignificant. The glomerular tufts did not fill the spaces nor project into the tubular orifices. There was often definite swelling and prominence of the parietal epithelium of the glomeruli and the epithelium covering the tuft was also somewhat swollen and unduly distinct. The endothelial cells of the glomerular capillaries were prominent and sometimes swollen, but there was no evidence of proliferation. Leucocytes were not increased and fibrin thrombi were absent. The glomerular basement membranes were of normal thickness. Owing to the prominence of the endothelial cells considerable difficulty is experienced in accepting any correlation of the degree of patency of the glomerular capillaries with the outline of the glomerular basement membrane. No other conclusion seems justified than that the circulation was adequate to maintain the nutrition of the related tubular epithelium.

Discussion

It is notoriously difficult to correlate defects of renal function with anatomical changes in the kidney, especially if the lesions are acute. In crush anuria the only significant change appears to be blockage of the more distal parts of the nephron with material deposited from the glomerular filtrate. The other changes, *i.e.* prominence of the parietal and visceral glomerular epithelium and slight swelling of the glomerular endothelial cells, might result from stasis of secretion within the nephron and from stasis of the glomerular circulation. In the relatively uncomplicated cases examined, the condition of the tubular epithelium suggests that, as in myelomatosis, no agent which is toxic in itself was present.

The changes somewhat resemble those found after an incompatible blood transfusion or the experimental administration of hæmoglobin to animals (lit., DeGowin *et al.*, 1938). Here also the reported incidence and degree of tubular necrosis are very variable, but the lesions have often been complicated by others

of a different kind. In both conditions the significance of inter-tubular œdema is very difficult to estimate.

Bywaters and Delory (1941) have found myohæmoglobin in the urine in two cases of crush anuria. The benzidine reaction appears too weak to indicate that the casts are homogeneous crystalline aggregates of this nature. It would suggest that this, or a very similar material derived from it, contributes to their formation. The molecular weight of myohæmoglobin is 17,500 (Svedberg, quoted by Milikan, 1939), i.e. about one quarter that of blood hæmoglobin. With this are associated a low renal threshold and the possibility of the rapid clearance of relatively large amounts of myohæmoglobin from the blood. Myohæmoglobin in the horse has an isoelectric point of 6.99 (Theorell, 1932), but its behaviour in the renal tubules is difficult to predict.

It has been suggested that in the crush syndrome the anuria is extrarenal in origin and results from a generalised shift of the body-water into the extra vascular spaces. These cases were not "drowned" by intravenous therapy and the distribution of the œdema fluid was local and not generalised. It was probably to be explained by diffusion of substances increasing capillary permeability from the crushed and broken down muscles. It should be emphasised that serious shock was absent and that, by the second day, blood pressure readings had returned to very satisfactory levels. Hypotheses based on deficient oxygenation of tissue appear untenable. In other cases the lesion may well be complicated.

All cases of anuria in air raid victims are not examples of this syndrome. In one case examined, almost every glomerulus was the seat of fat emboli, and in patients dying within a week other complicating factors are probably present. The specific gravity of the urine is low and not high, as in cardiac failure, where more marked œdema without uræmia is not infrequent. From the study of myelomatosis also it is apparent that œdema does not result from obstruction of renal tubules.

COMPARISON OF THE KIDNEY LESIONS OF MYELOMATOSIS AND CRUSH ANURIA

The casts in myelomatosis are much more prominent than those in crush anuria. They appear to fill the length of the tubules more completely, but only in the case reported by Holman did complete suppression of urine result. The rapidity with which obstruction develops plays a large part in determining the outcome of any lesion in the renal system. Provided they fill the lumen of a tubule at one level, the diameter and length to which casts may grow are not significant from the viewpoint of obstruction. In myelomatosis the formation of casts may well be intermittent.

and their increase in size must be gradual. Tubules so affected may allow the passage of urine and further casts will readily be deposited proximal to the partial obstruction. With the atrophy of obstructed tubules, especially if there is little fibrous tissue reaction, the still functioning tubules may become dilated and casts of relatively great diameter may be formed. In the crush syndrome the obstructing material, which is at least associated with a hæmoglobin derivative, is probably cleared from the blood almost as quickly as the circulation removes it from the damaged muscles. The conditions also may often be such as to favour concentration in the tubules. The material then readily precipitates out and aggregates into masses which become impacted, chiefly in the collecting tubules, as short-length casts. If the outflow of the related nephrons is not at first completely obstructed, further precipitation will complete the obstruction. In this acute condition, involving almost all tubules, it is very difficult for any tubule to undergo compensatory dilatation and, as fluid no longer passes down the affected tubules, the casts cannot increase further in size.

The lesions of crush anuria might thus be explained upon a purely obstructive basis. They are relatively acute. The extensive and more slowly formed aggregates in the tubules in myelomatosis and the other chronic changes found also in many cases of hydro-nephrosis—atrophy of tubules with inter-tubular increase of reticulum and fibrous tissue, changes in the capsular basement membrane and peri-glomerular tissues and regressive changes in the glomerular tufts—are not to be expected. In neither is there a primary tubular necrosis, but in both there is a reaction of the tubular epithelium at the site of the deposit in the lumen, and in those tubules whose function is interfered with there may be some secondary destruction and regeneration of epithelium. Tubules no longer able to function are removed and this may be attended with inflammatory-cell infiltration and with considerable diffuse increase of interstitial tissue.

SUMMARY

The renal changes associated with the deposition of casts in the renal tubules in cases of myelomatosis and of the crush syndrome are compared. In both, the primary importance of obstruction of the lumen is emphasised. Changes in the lining cells of the tubules are secondary to this obstruction rather than a primary degeneration. Glomerular changes are slight, but are consistent with a relatively small reduction of blood flow, insufficient in the great majority to produce marked ischæmic changes.

The chief difference lies in the slower deposition of the ultimately larger casts in cases of myelomatosis. This is associated with a

slower reduction of renal function. In the chronic condition there is also opportunity for removal of the tubular epithelium and this will be associated with collapse of the reticular framework, varying degrees of inflammatory cell infiltration, and reactive increase of fibrous tissue.

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ON THE FUNCTION OF TISSUE CELLS IN MEDIA
USED FOR GROWING VACCINIA VIRUS

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THE function of tissue cells in liquid media used for cultivating viruses has not been established. There are certain fundamental species and organ differences in tissues which are indicated by their ability or inability to promote the growth of a virus, but the nature of these differences cannot at present be stated in terms of physical or chemical properties. Such differences are reflected in the fact that viruses differ in the range of tissues with which they will grow. It is possible that all viruses do not depend upon the same requirements for growth derived from cells, but little is known of this subject and further studies of viruses from this point of view are required.

The conditions obtaining in culture have been investigated in relation to the growth of a number of viruses in attempting to establish some relation between properties of the medium and multiplication of virus. Thus information is available about the kind and amount of tissue, the duration of its viability as judged by its growth when transplanted to a fresh suitable medium, the composition of the liquid part of the medium, the hydrogen ion concentration and the oxidation-reduction potential, the respiratory activity of the tissue, the accessibility of oxygen to the tissue and the effect of plugging flasks with cotton wool or rubber stoppers. A good review of this subject is that of Hallauer (1938).

The experiments reported in this paper suggest a somewhat different approach to the investigation of the relation between tissue and multiplication of virus in so far as the results indicate that there are two parts to the problem, represented by two phases in the growth requirements of the virus. It appears that certain properties of the culture medium are required to initiate the growth of virus and that these properties are not necessarily the same as those which will suffice for multiplication of the virus in the later stages of a culture. These deductions apply at present only to the growth of vaccinia virus under the conditions of culture employed

in these experiments, but it is suggested that they might profitably be made a working hypothesis for similar investigations with other viruses or tissues.

METHODS

Three strains of vaccinia were used. Strains I and II were mentioned in an earlier paper (Maitland, Laing and Lyth, 1932). Strain I had been through 29-44 subcultures in rabbit tissue (usually kidney) in Tyrode or Tyrode-serum. Strain II was a strain of neurovaccine that had been cultured similarly after one animal passage in rabbit testis. Strain III was calf lymph from the Government laboratory and was cultured after one passage in rabbit testis.

The medium was made up in the proportion of 0.35-0.4 g. of minced rabbit kidney to 10 c.c. of Tyrode. The formula for the Tyrode's solution was given in the earlier paper (Maitland *et al.*, 1932). The mixture was inoculated in bulk and 2-2.5 c.c. were put in each of a number of Carrel flasks plugged with cotton wool and incubated at 37° C. in a moist atmosphere so that there was very little if any evaporation.

For titration the contents of one flask were ground in a mortar with sand and centrifuged to deposit visible particles. Five-fold dilutions of the supernatant fluid were made and 0.2 c.c. of each dilution injected into the shaved skin of a rabbit's back. The titre was taken as the highest dilution that caused a definite lesion.

EXPERIMENTAL RESULTS

In any one set of cultures the rate of growth of virus may not be the same in every flask and end-points of titrations may be affected by differences in the reactivity of rabbits. Conclusions have therefore been based upon similar experiments which have given consistent results.

Rate of growth of virus in culture

In table I the growth of virus is shown in terms of the titres of duplicate flasks of culture titrated immediately after inoculation and daily after incubation up to 5 days and at 7 days. The virus increased most rapidly during the first 2-3 days but there was continued growth after 3-4 days.

TABLE I
Growth of vaccinia virus in kidney and Tyrode's solution

Titre of virus in duplicate flasks after incubation for					
1 day	2 days	3 days	4 days	5 days	7 days
1 : 125	1 : 3125	1 : 15625	1 : 15625	1 : 15625	1 : 78125
1 : 125	1 : 15625	1 : 15625	1 : 78125	1 : 390625	1 : 78125

Titre before incubation 1 : 125.

In table II are shown the titres of single flasks of each of five sets of cultures after incubation up to 7 days. The titre before

incubation was calculated from the titre of the culture used as inoculum and its dilution on inoculation. Cultures A and C were the third and sixth subcultures of strain II, cultures B, D and E

TABLE II

Growth of 5 sets of cultures of vaccinia virus in kidney and Tyrode's solution

Designation of culture	Infective titre of virus in individual flasks after incubation for			
	2 days	4 days	5 days	7 days
A	1 125	1 78125		>1 390625
B	1 3125		1 15625	1 78125
C	1 625	1 3125		1 15625
D	1 25		1 3125	1 15625
E	1 25	1 3125		1 15625

Infective titre before incubation 1 125

were of strain I. The amount of tissue in culture E was about 0.25 g in 10 c.c., somewhat less than in the other cultures. These cultures, like those shown in table I, indicated growth of virus during the first 2-4 days and further increase after 4 or 5 days.

Increase of titre in the later stages of incubation was more strikingly evident in cultures with smaller amounts of tissue (table III). In experiments 125 and 126 with 0.024 and 0.036 g

TABLE III

Effect of amount of tissue on the growth of vaccinia virus in kidney and Tyrode's solution

Expt no	Amount of tissue in 10 c.c. of medium (g)	Infective titre of virus in separate flasks after incubation for				
		2 days	3 days	4 days	5 days	7 days
125	0.25	1 25		1 3125		1 15625
	0.098	1 25		1 3125		1 15625
	0.024	1 5		1 5		1 625
126	0.38	1 3125			1 15625	1 78125
	0.056	1 125			1 625	1 78125
	0.036	1 5			1 5	1 15625
127	0.25		1 3125		1 78125	1 15625
	0.064		1 625		1 15625	1 625
	0.018		1 5		1 5	1 5

Titre of virus before incubation 1 5

of kidney respectively there was no increase of virus (strain I) until after the 4th and 5th days. In experiment 127 the readings at 7 days may have indicated a fall in titre with the two larger amounts of tissue and failure of the virus to grow with the smallest

amount (0.018 g.) or they may have resulted from different reactivity of the rabbits used for titration. Taking the results of these three experiments together it is evident that with the larger amounts of tissue the virus multiplied considerably during the first 2 or 3 days and continued to increase after 4 or 5 days. Reduction in the amount of tissue tended to lengthen the period of lag and in two experiments there was no increase until after 4 or 5 days' incubation.

Incubation of the medium before inoculation

In contrast to the finding that virus could and did increase in cultures after 4 or 5 days, it appeared that virus did not grow if the medium had been incubated for 5 days or more before it was inoculated. Thus after about 5 days' incubation a culture that had been inoculated previously still provided conditions for the growth of virus, but uninoculated medium incubated for this length of time did not initiate growth of virus which was then added to it.

Strain I was inoculated into flasks of uninoculated medium and into others which had been incubated for various periods. Any loss of volume, which was small, was made up at the time of inoculation. The inoculated flasks were then incubated for further periods of 2-17 days to give time for growth of virus before the contents were titrated. The results of two such experiments are shown in table IV. As a base line the contents of a flask just after inoculation were titrated at the beginning of each experiment. In controls inoculated without preliminary incubation and in medium incubated for 2 days (expt. 61) and for 3 days (expt. 59) before inoculation the virus grew. In medium incubated for 5 days or more and then inoculated the virus did not grow. The slightly higher titre in two flasks inoculated after incubation for 5 days (expt. 59) could have been within the limits of the error of the technique but might have indicated slight growth of virus. The behaviour of virus in flasks incubated for 5 days or more before inoculation was however in clear contrast to the definite multiplication that occurred in flasks inoculated without preliminary incubation or after incubation for 2-3 days.

Thus with regard to preliminary incubation there was a period of at least three days during which inoculation of virus resulted in its multiplication. This period came to an end gradually and virus inoculated at five days either failed to grow or grew very little; after five days the inoculated virus did not grow. These time relationships refer only to the experimental conditions employed but they indicate a conclusion which may be stated in general terms. After a period of preliminary incubation the medium lost its property to initiate growth of virus but it still promoted growth of virus which was already in the medium. Thus

TABLE IV

Growth of vaccinia virus in medium inoculated after preliminary incubation

Time in days of incubation of medium before inoculation	Titre of individual flasks at intervals (in days) after inoculation										
	2	3	4	5	7	10	11	12	14	17	
Experiment 59 Titre when inoculated 1 125											
0					1 15625	1 15625		1 125	1 3125	1 15625	
3			1 3125		1 125		1 78125		1 3125		
5				1 625				1 625	1 125		
7		1 5		1 25	1 5			1 5			
10	1 5		1 125		1 625	1 5	1 125				
Experiment 61 Titre when inoculated 1 625											
	4	5	7	9	12	14	16				
0					1 78125	1 78125		1 3125			
2			1 15625			1 3125	1 125				
5	1 125		1 5	1 25							
7	.	1 625	1 125	1 125							

there were two properties of the medium related to the growth of virus. The first property was required to initiate growth. Virus did not grow unless the medium was inoculated before preliminary incubation had removed some essential property upon which the initiation of growth depended. The cultures with small amounts of tissue were instructive on this point (table III) for there was a long lag. In these cultures the virus did not increase until the growth-initiating property of the medium had been greatly weakened or lost; the medium had however been inoculated when this property existed fully.

The second property of the medium was revealed by increase of virus at a time when the medium would no longer initiate growth. It was evident that growth-initiating and growth-promoting properties existed concurrently when the medium was fresh and that during incubation the growth-initiating property was lost first. Whether the growth-promoting property was the same at the beginning of a culture before the growth-initiating property was lost as it was later on after the growth-initiating property had disappeared is an open question. It might therefore be profitable to separate these properties of the medium for the purpose of analysing the relation between the functions of cells and multiplication of virus. With this point of view in mind a few further observations have been made.

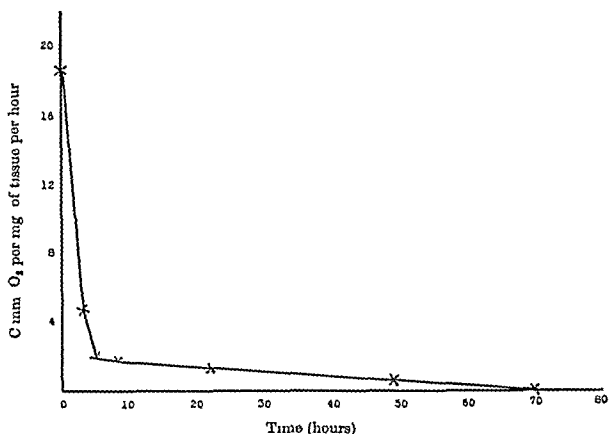
Respiration of tissue incubated under conditions of culture

Thin slices of kidney were incubated in Tyrode in Carrel flasks at 37° C. From time to time tissue was removed and resuspended in Tyrode and its rate of utilising oxygen was measured according to the technique of Warburg. A typical result, shown in the figure, conformed to what is well known. The respiratory activity fell rapidly during the first few hours and thereafter more slowly and gradually came to an end in about 3 days. Minced kidney gave similar results but tended to stop respiring somewhat earlier than thin slices.

There appeared to be no correlation between the amount of tissue respiration and the growth-initiating property of the medium. Virus grew well whether the medium was inoculated when it was freshly prepared and respiration was active or after incubation for 2-3 days when respiratory activity was very small. Whether the growth-initiating property might have been co-existent with a certain minimal degree of tissue respiration was more difficult to decide. In the later stages of a culture, growth of virus did not correlate with measurable tissue respiration.

Treatment of tissue with cyanide and with water

Preliminary treatment of the tissue with cyanide abolished the growth initiating property of the medium. About 0.304 g of minced kidney was suspended in 3 c.c. of Tyrode's solution containing $M/100$ KCN and left in a shallow layer at room temperature for 3.4 hours. The tissue was then washed thoroughly in Tyrode and made up as medium with fresh Tyrode. It was inoculated and incubated as in the earlier experiments. Under these conditions the virus did not grow. Tissue put through the same process with Tyrode in the absence of cyanide permitted growth of the virus, and virus survived as well at 37°C in Tyrode containing $M/1000$.



Oxygen uptake of minced rabbit kidney after incubation in Tyrode solution for various periods

cyanide as in Tyrode alone. From this result it did not necessarily follow that inhibition of the cyanide sensitive respiratory processes of the tissue was directly responsible for the loss of its growth-initiating property. Disorganisation of the chemical processes of the cell rather than a particular change caused by treatment with cyanide might have been the essential feature. Disorganisation of the cell produced by other means was equally effective.

Tissue treated with water lost its growth initiating property. Several experiments were made using strain I in the 42nd and 43rd subcultures, strain II in the 22nd and 23rd subcultures and strain III in the 3rd subculture. About 0.304 g of minced kidney was added to 3.5 c.c. of sterile distilled or tap water and left in

a shallow layer at room temperature for 2-4 hours. In making up the medium one of three procedures was then followed: (a) the liquid was removed, the tissue washed well in Tyrode and made up to 10 c.c. with fresh Tyrode: (b) nothing was removed and concentrated Tyrode was added to make a final volume of 10 c.c. of properly constituted Tyrode's solution: (c) as for (b) with the addition of 20 per cent. of rabbit serum. Inoculation and incubation were carried out as stated. Titrations were made at intervals between 18 hours and 7 days of incubation. In some experiments the contents of two flasks were pooled for titration to smooth out any irregularities in the amount of growth of virus in individual flasks.

For each experiment a control was put up with tissue soaked in Tyrode and otherwise treated similarly. In these controls the virus grew. On no occasion when the tissue had been treated with water did it suffice as a medium for growth of virus. In some experiments nothing was removed so that the loss of the growth-initiating property of the medium could only be attributed to disorganisation of the cells by soaking them in water. The addition of serum to the medium did not compensate for the damage to the tissue sustained while in water.

Treatment of the tissue with water caused a marked fall in its respiratory activity. One set of observations was made with sliced rabbit kidney cortex. The oxygen utilised, expressed as c.mm. oxygen per mg. dry weight of tissue per hour was, for fresh tissue, 20.9; after soaking in Tyrode for 2 hours, 13.7; after being in distilled water for 2 hours, 1.6. This marked fall in respiratory activity did not result from interference with any particular chemical reaction as such. It appeared rather to indicate the loss of chemical activity which had been associated with structural or physical integrity of the cell. The fall of respiratory activity after treatment with water might therefore be regarded as an indicator of damage sustained by the cell and the loss of growth-initiating property might be regarded as a consequence of this damage.

DISCUSSION

The view here put forward about the relation of tissue cells to the growth of vaccinia virus in a liquid medium is that there is in the first place some essential function of the cell which is necessary to initiate growth. This function appears to depend upon a certain integrity of the cell as a cell and it can be abolished in several ways. In the experiments described in this paper it has been abolished by treatment with cyanide, by soaking in water and by incubation at 37° C. in Tyrode's solution. Each of these procedures markedly reduces the respiratory activity of the cells

but presumably through different kinds of harm done to them, and it is therefore concluded that diminished respiratory activity is not necessarily directly responsible for the loss of the growth-initiating function of the tissue. The failure of the tissue to initiate virus growth is attributed to cell damage of one kind or another, of which the fall in respiration could be regarded as an indicator, although it is not possible at present to do more than speculate on the chemical processes of the cell which are essential for initiating growth of virus. Other ways of making cells unsuitable for growing vaccinia virus have been reported, for instance freezing and thawing (Rivers *et al.*, 1929) and heating at 45° C. for 20 minutes (Maitland and Laing, 1930). Breinl (1932-33) found that heating tissue at 45° C. for 30 minutes or incubating it at 37° C. for 3 days or treating it with cyanide prevented it from functioning as a medium for the growth of vaccinia virus in Tyrode's solution and serum. Zinsser and Schoenbach (1937) noted that tissue heated at 50° C. for 10 minutes failed to grow virus.

The first part of the problem thus resolves itself into determining the property of cells that is necessary for initiating growth of virus and that is lost concurrently with cell damage produced in several ways. There is evidence that vaccinia virus very rapidly becomes attached to tissue cells and enters into so close an association with them that it is protected from the action of antibodies. Rous *et al.* (1935) showed among other things that, although killed cells fixed virus, they did not protect it from the action of immune serum as did living cells. They stated that "the protection of the viruses is in some way dependent upon cell life". The property of living cells which is concerned in the protection of virus may also be required to initiate growth of virus. Further work is needed to elucidate more precisely what happens in either event. Rapid fixation and protection by living cells have been demonstrated for other viruses, including the Shope fibroma virus (Rous *et al.*), which indicates a general phenomenon in cell-virus relationship and gives added point to the suggestion that it may apply to culture. There is ample evidence for vaccinia and some other viruses that intracellular growth occurs and that in cultures, although virus may be in the suspending medium, much of it is closely attached to the tissue. Vaccinia grown in tissue spread on a slant of special agar appeared to be entirely associated with the cells (Kurotchkin, 1939). We have grown strain II in minced kidney suspended in 0.85 per cent. NaCl and have carried it through several subcultures. There was no significant difference between the titre of cultures in this medium and that of parallel cultures in minced kidney and Tyrode's solution. We failed to grow the virus in extracts of kidney in Tyrode's solution (Maitland *et al.*), and it is unlikely that the virus grew in the liquid part of the

sodium chloride medium, though some additional substances were no doubt added to it from the tissue. All the evidence indicating that virus grows in or on cells in liquid media accords with the view that some essential association between virus and cell is required to initiate growth.

The further interesting fact is that, if the requirements for initiating growth of virus have been fulfilled, the virus will multiply when these conditions no longer obtain, and this implies that the mechanism which will suffice for continued growth need not be the same as that required to start it. It may be that the essential prerequisite for growth is made available by the combination of virus with a living or surviving cell; there is then a variable period before growth of virus is evident, and in some circumstances the increase of virus may be delayed until conditions which would fulfil the prerequisite requirements have disappeared. The time relationships of the growth of virus may be in part the outcome of the experimental method used to detect growth. Grinding up tissue in order to release virus and titrating infectivity may fail to give correlation with the microscopic changes in the cells which indicate multiplication of virus as described in the studies of Bland and Robinow (1939) and other authors whose work they review. Nevertheless conclusions which are equally valid may be drawn from the type of experiment recorded in this paper. The second part of the problem is therefore to elucidate what is required to promote the actual multiplication of virus and to determine whether this is the same in the early as in the later phases of a culture.

Zinsser and Schoenbach (1937) deduced that there was a fundamental difference between the virus of equine encephalitis (western type) and *Rickettsia* on the basis of growth requirements. They stated (p. 207) "a fundamental physiological difference between the growth requirements of the two classes of infectious agents is indicated by the fact that, whereas virus growth reaches its maximum usually between the 3rd and 4th days, at a time when . . . the tissue is still alive, *Rickettsia* multiplication appears to speed up after the 6th, 7th or 8th day when presumably tissue viability and, consequently, metabolism have come to a stand-still". According to the view presented in the foregoing discussion it would be fundamental to determine whether the two classes of infectious agent require similar or different conditions to initiate growth, and to this question the experiments of Zinsser and Schoenbach do not afford an answer. In the later work of Zinsser *et al.* (1939), when they used as medium a slant of special agar on which minced tissue was spread and on which the cells might perhaps survive for a shorter time than in a liquid medium (though there is no evidence on this point), the tissue was inoculated while it

was fresh Zinsser and Schoenbach noted (p 223) that "Rickettsiae can be grown on tissue cultures which are not inoculated until after a preliminary incubation of 3 or 4 days" They stated further that in such cultures the Rickettsiae invaded and multiplied in the presumably inactive cells, and although their growth did not depend upon the vital activities of the cells they still appeared to require conditions established within the inactive cells before cytoplasmic deterioration had made much progress Thus in these experiments there is an implication that Rickettsia will not grow unless the medium is inoculated before some growth-initiating property is lost If this should be so, a resemblance to vaccinia could be stressed rather than a difference based upon a longer lag period before growth develops, for vaccinia under some circumstances may have a long lag Investigations along the lines here indicated and with other viruses would be profitable in revealing similarities or differences in the nature of their growth requirements

SUMMARY

The problem of assessing the function of cells in promoting the growth of vaccinia virus in a medium of minced rabbit kidney and Tyrode's solution may be considered in two parts The first concerns the property of cells necessary to initiate growth of virus This property can be abolished in several ways which damage cells and may be similar to that property of living cells which protects virus from the action of antibody When the growth initiating function of the medium has had effect, virus will continue to grow after this property has been lost The mechanism required to initiate virus growth and that which will suffice for the continuation of growth are not necessarily the same

We are indebted to Dr D G Evans for the measurement of tissue respiration

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E. B.

576 . 809 . 734 : 576 . 851 . 4 (*Bact typhosum*)

DARK GROUND STUDIES OF VI AGGLUTINATION OF *B. TYPHOSUS**

ADRIANUS PIJPER

From the author's private laboratory, Pretoria, South Africa

(PLATES XXXI XXXIII)

No objections have been raised to the statement of Felix and Pitt (1934, p 187) that "The macroscopic appearance of the Vi agglutination is very similar to that of O agglutination" Microscopically however I have found the whole process of Vi agglutination, including its end results, very different from O agglutination and also from H agglutination The microscopic characteristics of the three kinds of agglutination can only be properly studied by making the motile organs of the typhoid bacillus visible in full activity The points of difference are best appreciated by direct comparison

Motile organs of the typhoid bacillus

These can be seen and photographed in activity by means of dark ground methods with the sun as light source (Pijper, 1930, 1931-32, 1938, 1940, 1941) Briefly my technique involves the use of sunlight, a helostat, a 2-metre focus collector lens, a Siedentopf cardioid condenser and Zeiss apochromat objectives $\times 35$ or $\times 60$, but requires no coolers, no filters and no additional mirrors A Kontax camera with Kodak Super XX film allows sufficiently short exposures Mica-glass slides provide the best black background † Typhoid bacilli are then seen to be propelled by a long straight tail in the direction of their long axis (fig 1) The tail is a long-drawn out spiral Slowing down is accompanied by a broadening of the spiral (fig 2), which, when the organism is at rest, may unwind into its two constituent flagella (fig 3) These are attached to the side of the bacillus Restarting, the flagella begin to revolve again in spiral fashion and so propel the bacillus, which rotates round its long axis The two flagella become twisted round one another and form the very elongated spiral which constitutes the

* For the investigations a grant was received from the Research Grant Board of the Union of South Africa

† These slides, originally made by Zeiss can now also be obtained from G F Gurr, 136 New King's Road, Fulham, London

tail (fig. 1). The black gap in fig. 1 between body and tail is caused by the two flagella being separated at that spot and not in focus. The twisting and untwisting of the flagella is a reversible process.

For completeness it should be added that whilst at rest the two flagella can sometimes be seen to untwist into a large number of fine threads, which wave about and gradually disappear. This process is not reversible.

The effect of drying, fixing and staining on the distribution of flagella and the concomitant production of artefacts were described previously (Pijper, 1938).

H agglutination

In previous publications (Pijper, 1938, 1941) it was shown that the specific effect of *H* agglutinating serum is that the tails (or the two flagella) and the bodies of the bacilli become covered with granules which gradually coalesce and form a continuous sheath. This thickening of tails and flagella impedes motion. The motile organs appear as thick corkscrews attached to thickened bacillary bodies and there are never more than two per bacillary body. Motility gets less and less and there is no sign of mutual attraction. Slight motility and currents bring corkscrews into contact with one another, and sometimes they get entangled. This fortuitous entanglement appears as agglutination but it is obvious that it is a purely mechanical result of the specific thickening of the tails and flagella. The origin of the granules forming the sheaths is unknown. In the final stages one sees a number of thickened bacilli held together by intertwined thick wavy cables.

O agglutination

In previous papers (Pijper, 1938, 1941) it was shown that the addition of *O* agglutinating serum to freely motile typhoid bacilli completely alters their conduct towards each other. Normally they never collide, but now they begin to approach one another at high speed. Repulsion has apparently changed into attraction. They become and remain attached to one another in polar fashion. The process reminds one of crystallisation. The large clumps, which are gradually built up by more and more bacilli attaching themselves head-first to the periphery of smaller clumps, acquire a definite and typical architectural structure. Nearly all attachments are end-to-side or end-to-end. This produces a characteristic pattern.

Vi agglutination

Vi agglutinating serum was prepared in rabbits by injections of formal-treated typhoid bacilli. *Vi* agglutinating sera of other origin were also used. Great care was always taken to have the other agglutinins completely absorbed before a serum was used for the dark-ground observations.

THE VI AGGLUTINATION PROCESS



FIG. 1—Young typhoid bacilli, swimming fast and showing tails. $\times 1000$



FIG. 2—Old typhoid bacillus, resting, showing spiral nature of tail $\times 1000$

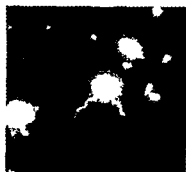


FIG. 3.—Old typhoid bacillus, resting, having the tail untwisted into two flagella $\times 1000$.



FIG. 4—Young typhoid bacillus, suddenly affected by Vi agglutinating serum $\times 1000$



FIG. 5—Young typhoid bacilli tails affected by Vi agglutinating serum $\times 1000$



FIG. 6—Young typhoid bacillus, tail gone limp through action of Vi agglutinating serum. $\times 1000$



FIG. 7—Beginnings of Vi agglutination $\times 600$



FIG. 8—Small Vi agglutination clumps—side to side attachment noticeable > 1000



FIG. 9.—Small Vi agglutination clump: typical attachment of bacilli. $\times 1500$

As a rule a drop of diluted Vi agglutinating serum was placed on a microscope slide and the drop lightly touched with a small loopful of a fresh broth culture of the bacilli. A coverglass was then sealed down with vaseline. For mass observations the hanging drop method was used occasionally. Most of the observations were made on Felix's strain of typhoid bacilli Ty 2. Bhatnagar's strain Vi I was also used.

The change in the tail of typhoid bacilli from a long drawn out spiral (fig 1) into a broad spiral (fig 2) when movement slows down is rare in young cultures. It occurs with increasing frequency as the culture ages, and it can usually be seen in a few bacilli when a preparation between slide and coverslip is left on the stage of the microscope for more than 24 hours. Even then it has to be searched for.

Now it is one of the most striking features of the effect of a Vi agglutinating serum on freely motile typhoid bacilli that the bacilli exhibit this phenomenon of slowing down, accompanied by broadening of the spiral, very generally and very rapidly. It becomes a very impressive phenomenon because it looks as if a large number of bacilli had suddenly become afflicted with a sort of paresis of their motile organs. Tails rotate less rapidly, motion is less energetic, erratic side movements take place and some bacilli even move backwards and swim tail foremost. Gone are the straight tails, broad spirals have taken their place. It is as if a sudden fatigue had come over a large number of individuals. Figs 4 and 5 show typical examples. Temporary recovery from this "fatigue" or "paresis" sometimes takes place, and then the tail straightens out again and ordinary movement is restored. But it does not take long before a bacillus that has once been affected again exhibits this sudden paresis. The whole picture is different from what one may see in an old culture in the absence of serum in that here it is much more wide spread and much more sudden. Also, in Vi agglutination the paresis often goes further and the tail may become limp and lose its appearance of a stiff coil, as in fig 6.

In some cases the tail first shows the symptoms of paresis and then untwists into the two flagella, which then keep rotating in a very irregular fashion, again resulting in very erratic movements of the bacillus.

These erratic movements, caused by the increasing lameness of the motile organs, remind one somewhat of the first stage of H agglutination, where somewhat similar movements are performed. But in H agglutination these movements are obviously caused by the enormous thickening of the motile organs, in Vi agglutination I have so far failed to notice any such thickening. The phenomenon in Vi agglutination, in its suddenness, wide spread occurrence and unexplainedness, is all the more dramatic.

It must be added that not all the bacilli in a given preparation are affected to the same extent. Some seem to escape entirely, others merely become somewhat fitful in their movements. The general appearance of the preparation, however, differs very markedly from that of normal bacilli. At a time when control preparations of normal bacilli in broth still show a preponderance of bacilli going through the field in straight lines, most of the bacilli in Vi serum exhibit the typical erratic movements.

It is these spasmodic erratic movements which, in combination with another factor, bring about agglutination. It must be remembered that normal typhoid bacilli, whatever their speed, practically always succeed in avoiding collisions. The irregular movements which form the first stage of Vi agglutination lead to rather frequent collisions. There is no evidence that the repellent forces which normally operate between bacilli have disappeared. They may have become less powerful, but one often sees occurrences which indicate that the bacilli still repel one another. There is certainly no trace of mutual attraction, which was shown to be the leading feature of O agglutination, but the erratic movements force the bacilli up against one another. The factor which governs mutual repulsion is apparently not always strong enough to overcome the driving force of the irregular action of the affected tails.

At first collisions are still rare but they gradually increase in number. It then happens that the colliding bacilli remain attached to one another and this is the beginning of agglutination. The attachment however is not always permanent. Often the bacilli through the same spasmodic movements free themselves again. The outcome largely depends on how the collision takes place. The larger the surface areas that come into contact, the greater is the chance that the union will be permanent. From this one must conclude that the surface of the bacilli has become sticky. Extensive contact would then make it more difficult for bacilli that still have some motility left to break away.

There are therefore three factors to be considered in Vi agglutination: erratic movement, mutual repulsion, and stickiness of surface. The interplay of these three factors explains the peculiar pattern of Vi agglutination clumps. End-to-end attachments do occur, but they are often not permanent and therefore remain rare. Side-to-side attachments are naturally more permanent and therefore prevail.

Fig. 7 illustrates an early stage in the formation of clumps, and is of particular interest. Its pattern has a superficial resemblance to that of O agglutination; it looks somewhat like polar attachments. Actually the lower two bacilli were forced up against the long thread and remained stuck. What looks like end-to-side

THE V_i AGGLUTINATION PROCESS



FIG 10—Two V_i agglutination clumps typical pattern
× 600



FIG 11 Small V_i agglutination
clump showing tails or flagella
at periphery × 1000



FIG 12—Large V_i agglutination clump typical pattern
× 1000

attachment is in reality side-to-end attachment. Fig. 8 shows the more common side-to-side attachment in small groups. Fig. 9 is a typical example of the prevalent side-to-side method of linking up.

Such small groups often arise very gradually. It often happens that bacilli keep on performing spasmodic movements around a couple of bacilli that have become joined together, without ever making real contact. Or they may become attached by one pole and get away again when their motive power overcomes the forces of adhesion. The growth of groups is a very tedious process to watch. Hours may elapse without any significant growth taking place. It is obvious that the speed of growth is dependent on the number of bacilli that can take part. Growth is therefore particularly slow in slide and coverslip preparations, faster in hanging drops and at its best in tubes, for slide and coverslip preparations have to be kept thin and the number of bacilli low, otherwise the picture becomes confusing. Fig. 10 shows the kind of groups formed under the microscope at room temperature in about 24 hours. Fig. 11 is a similar group, purposely overexposed so as to bring out the tails or flagella, which do not appear in the other pictures. The latter aimed merely at bringing out the outlines of the bacilli. It is clearly seen in fig. 11 that the tails and flagella take no part in constructing the clump.

Figs. 12, 13 and 14 are pictures of the larger agglutination groups formed in agglutination tubes and then transferred to a microscope slide. They show the typical pattern of *V1* agglutination groups: the side-to-side attachment predominates and gives the clump its characteristic appearance.

Occasionally it happens that, through the erratic movements described above, the tails or flagella of several bacilli become entangled, but this is rare. As a rule also it is not a lasting condition, the slight motility left is usually enough to bring such bacilli into the more common close contact.

With the non-motile *V1* strain in *V1* agglutinating serum the first stage of *V1* agglutination—the increasing paresis of the motile organs—is of course absent. Slight currents in the surrounding fluid and Brownian movement are the only forces that bring the bacilli together and thus makes clumping a very slow process. The principles however that govern *V1* agglutination remain the same. Once bacilli have been brought into close contact, there is a tendency to stick together. Here too the greater the area of contact the greater does the adhesive force turn out to be. The process is slow, but contact once established is less likely to be disrupted than in the case of motile bacilli. The pattern of the clumps formed is the same as with motile bacilli—a preponderance of side to side attachments.

SUMMARY

Dark-ground microscopic studies of the three types of agglutination of which typhoid bacilli are capable (H, O and Vi) have been made with the sun as the source of light. Sunlight is the only light source that will make the motile organs visible and capable of being photographed. In this way the following points have been demonstrated.

1. In H agglutination the specific action of the serum is to cover the tails and flagella with a sheath. The thickened motile organs then get entangled mechanically.

2. In O agglutination the bacilli no longer repel but become attracted towards one another. They approach each other head first, become attached in polar fashion and build up characteristic patterns resembling crystals.

3. In Vi agglutination there are three factors: a growing paresis of motile organs, the normal repulsion between bacilli and a stickiness of the surface of the bacilli. The paresis causes erratic movements, which overcome the normal repulsion, and the stickiness makes accidental contacts permanent. Side-to-side attachments prevail.

It is curious that of the three processes the Vi agglutination process, the last to be discovered, is the only one for which the word "agglutination" in the literal sense is really appropriate.

Note. Three 16-mm. microcinematographic films, made to illustrate and support the views expressed in this and previous papers, are now available.

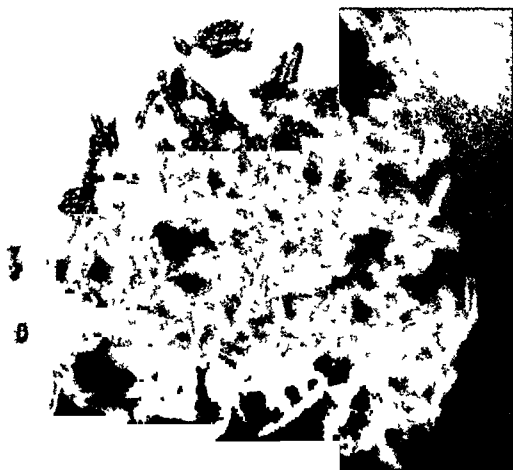
1. Sunlight dark-ground technique and motility of typhoid bacilli. 400 ft.
2. O agglutination of typhoid bacilli. 400 ft.
3. H agglutination of typhoid bacilli. 700 ft.

Duplicates of these can be had from the author at cost price. A film dealing with Vi agglutination is in course of preparation.

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FIG. VI AGGLUTINATION PROCESS



Figs 13 and 14 —Large Vi agglutination clumps typical pattern $\times 1000$

SHORT ARTICLES

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METASTASISING BASAL CELL CARCINOMA

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(PLATES XXXIV-XXXVI)

Metastases from basal cell carcinoma are extremely rare and, when they occur, are usually confined to the regional lymph glands. Willis (1934), who reviewed the literature, accepted only three examples and there have been no further records since then. The present case is thought worthy of report because of several original features: (1) the unequivocal appearance of basal cell carcinoma presented by the tumour, (2) the widespread metastases in bones and lungs proved to be blood borne; (3) a relatively benign clinical course over a period of many years, (4) the production of leuco erythroblastic anaemia.

CASE REPORT

Amy F., aged 49, a housewife, was admitted in December 1938 for pains in the back and weakness. The family history was irrelevant. She had been quite well until March 1933, when she noticed that a small lump in the middle of the forehead, which she said had always been present, had begun to enlarge and had reached the size of a "pea" within six months. A diagnosis of inflammatory lesion was made and the lump was incised in October 1933, but in March 1934 it had recurred and had reached the same size. In October 1934 the lump was again incised, but six months later it had returned and it was incised once more in October 1935, when the wound suppurated. When the suppuration had subsided, the patient noticed that two smaller but similar lumps were present in the same position, these gradually enlarged and fused. She was then admitted to Guy's Hospital for the first time, when a tumour measuring 2.5×2 cm, slightly raised and fixed to both skin and underlying bone, was present in the middle of the forehead. The tumour was completely excised and on histological examination proved to be a basal cell carcinoma (fig. 1 *). She was discharged free from symptoms and subsequently received a course of deep X ray therapy. She had no further trouble until July 1938, when, after a short period of weakness, she complained of severe pain across the back, which soon spread to the ribs and shoulders. The pain was accompanied by sensations of pressure in the sternum, which was painful on respiration. These pains became progressively more severe and eventually spread to the thighs and legs. Meanwhile she had become increasingly breathless on exertion. She had lost six kg. in weight in six months.

On examination, the patient lay very still. She was extremely pale, but apart from a transverse surgical scar across her forehead showed no

* This was diagnosed as a basal cell carcinoma by Professor G. W. de P. Nicholson, who also confirmed the diagnosis in the subsequent secondary deposits

other external abnormality. No abnormality was detected in the cardiovascular, respiratory, alimentary, genito-urinary or nervous system. In the skeletal system, the ribs, sternum, dorsal vertebrae, left humerus and both tibiae were very painful on palpation.

Radiological investigations. Radioscopy of the skeleton showed numerous small rounded translucent areas, suggestive of metastatic deposits, in the spine, ribs, scapulae, pelvis and upper ends of both humeri.

Blood picture. The haemoglobin was 45 per cent. The red blood cells were 2.3 million, the white cells 6700 per c.mm. The differential leucocyte count showed polymorphonuclear neutrophils 39, lymphocytes 27, hyaline cells 11, metamyelocytes 2, myelocytes 8, metamyeloblasts 12 and myeloblasts 1 per cent. The red cells showed moderate anisocytosis and poikilocytosis. Nucleated red cells were numerous (1700 per c.mm.) and several showed mitosis and pink-stippled cytoplasm. Reticulocytes were 12 per cent., platelets 23,000 per c.mm. A second haematological examination a month later revealed little change.

She was treated symptomatically with analgesics and died two months after admission.

Autopsy (P.M. 54/1939)

This was performed 22 hours after death.

External appearances. The body was that of an elderly female of slight build, showing little subcutaneous fat, marked pallor of the skin and no other abnormality except an old healed surgical scar on the forehead.

Cardiovascular system. The heart (280 g.) was normal except for senile changes, which were present also in the aorta.

Respiratory system. The larynx, trachea and main bronchi were normal. The pleural cavities were dry and the pleural membranes normal. Both lungs showed numerous white subpleural deposits, which were slightly raised, firm and rounded and measured less than 3×1 mm. The remainder of the lungs was normal.

Alimentary system. No abnormality was detected and both mesentery and peritoneum were normal.

Hepatic system. Except for a small subcapsular cavernous haemangioma, the liver was normal. The gall bladder, biliary passages and pancreas were normal.

Lymphatic system. The spleen (520 g.) was enlarged, with soft greyish red pulp, and a small infarct in its lower pole. The cervical, mediastinal, mesenteric, para-aortic and inguinal lymph glands were normal.

Urinary system. No abnormality was detected in the kidneys, ureters or bladder.

Genital system. The breasts were normal. The right ovary showed a small follicular cyst filled with clear fluid. The left ovary, Fallopian tubes, uterus and vagina were normal.

Endocrine system. Adrenal glands and thyroid were normal.

Skeletal system. The sternum showed numerous large rounded deposits which protruded from the marrow and produced dome-shaped elevations of the periosteum of the posterior surface. The right humerus showed diffuse infiltration of the entire length of the marrow with firm, white tissue similar to the masses in the sternum.

Histological examination

Sections of the primary tumour removed in 1936 showed a typical basal cell carcinoma (figs. 1 and 2) arising from the epidermis and infiltrating the dermis as far as, but not involving, the underlying striped muscle. The

METASTASISING BASAL CELL CARCINOMA



FIG. 1—Section of primary tumour of the forehead showing typical morphology of basal cell carcinoma. H. and E. $\times 80$.

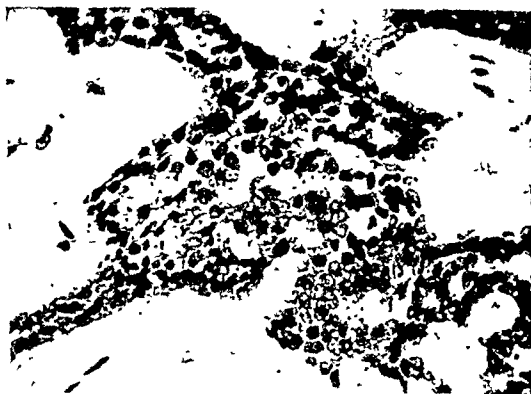


FIG. 2—High power view from same section as fig. 1. H. and E. $\times 320$.

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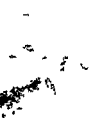
METASTASISING BASAL CELL CARCINOMA



FIG. 3.—Section of lung showing small metastasis in an anthracotic nodule. H and E. $\times 80$



FIG. 4.—Section of bone (sternum) showing metastases. H. and E. $\times 80$.



METASTASISING BASAL CELL CARCINOMA

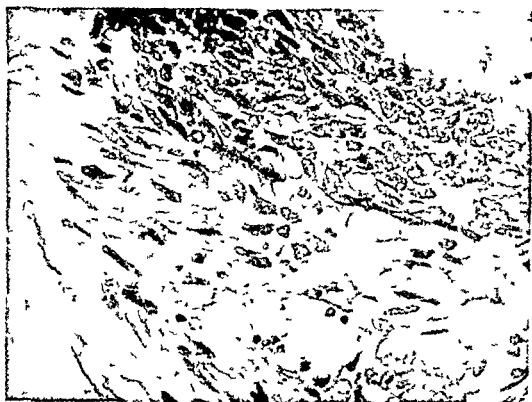


FIG 5—High power view of fig 4 H and L 320

FIG 6—Section from sternum showing tumour nodule in lumen of blood vessel H and E $\times 80$

tumour was composed for the most part of small, spheroidal, hyperchromatic cells of uniform size and shape, arranged in compact ramifying strands. No mitoses were seen. The skin was ulcerated.

The lungs showed small flat subpleural metastases similar in morphology to the primary (fig. 3). Early bronchopneumonia was present. The sternum, humerus and vertebrae showed numerous metastases which in many sections completely filled the marrow spaces and lacunae and in many areas had infiltrated the whole thickness of the cortical bone, perforating the periosteum. There was a tendency for the growth to follow the contours of the marrow spaces (fig. 4) and the morphology remained similar to that of the primary tumour (fig. 5). Besides replacement of bone in the areas of tumour formation, there was considerable resorption and fibrosis of the bony trabeculae. The marrow was too scanty for adequate examination. In the course of serial sections, one was fortunate in finding a small mass of tumour cells in a blood vessel (fig. 6). The spleen showed almost complete atrophy of the lymphoid tissue, though the sinuses were filled with an excess of cells, a moderate number of which were nucleated red cells and undifferentiated leucocytes. There was also a small ischaemic infarct. The kidneys, breasts, thyroid, adrenal glands and ovaries were normal.

DISCUSSION

It is well known that transitional types exist between the typical basal cell carcinoma and squamous epithelioma and, since both presumably arise from the same germinal layer, this is not unexpected, though these transitional types usually show some degree of differentiation in either the primary tumour or its metastases. Of the cases reported as metastasising basal cell carcinoma, the metastases in Niles's (1931) case showed typical squamous cell carcinoma and the photomicrograph of the primary strongly suggests a transitional cell type. Similarly the case reported by Spies (1930), with generalised metastases, may be an adenoid cystic epithelioma. While there cannot be any strict definition of basal cell carcinoma, the morphology is usually sufficiently characteristic to render its recognition certain. The example presented in this paper is quite typical morphologically and the metastases showed no deviation in the degree of differentiation from the primary tumour.

A consideration of the mode of spread reveals interesting features. The tumour arising in the skin of the forehead showed no evidence of metastasis to the regional lymph glands, but the presence of secondary deposits in lungs and bones clearly indicates blood borne dissemination and the demonstration of tumour tissue within a blood vessel of the sternum (fig. 4) confirms this so far as is possible with the methods available. The widespread skeletal dissemination in itself suggests systemic arterial embolism, after passage through the lungs.

SUMMARY

A case of long standing basal cell carcinoma with metastases is described. Evidence is presented which suggests that the metastases were blood borne.

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ON THE ORIGIN OF BACTERIOPHAGES FOR THE
LACTIC STREPTOCOCCI

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In a previous paper (Whitehead and Hunter, 1937) on the development of bacteriophage in cultures of lactic streptococci, evidence was brought forward in favour of the hypothesis that the phage could originate within the culture itself. Subsequent work has shown that this hypothesis is no longer tenable and that many of the observations on which it was based can be explained satisfactorily on the more prosaic basis of contamination of the cultures with bacteriophage from external sources. Most of the new observations have been described elsewhere in detail (Whitehead and Hunter, 1941), but it is thought desirable to summarise here the significant points.

(a) A strong concentration of air-borne bacteriophage is commonly found in the surroundings of commercial dairy factories and occasionally in laboratories where cultures of lactic streptococci are carried; this was quite unsuspected in 1937. The presence of air-borne phage makes the risk of contamination of cultures with phage during transference to a new batch of medium very great at certain periods. Instances have been observed where the removal of a cotton wool plug from a vessel of sterilised milk for five seconds has admitted sufficient phage from the surrounding atmosphere to cause lysis of a culture in the milk within eighteen hours. The risk of contamination is greater in the preparation of a large bulk of culture and increases with time of exposure of the culture medium to the atmosphere after heat sterilisation. The frequent occurrence of air-borne phage in laboratories is, one would judge from the literature, not generally appreciated. It is our experience that the occurrence of phage in the air is spasmodic and that it may possibly be determined by such factors as humidity, temperature, and wind direction and strength.

(b) The immediate origin of phage in cheese factories is most probably the cheese whey. The contents of the vats (700-1000 gallon capacity) during the process of cheese manufacture are in effect large exposed cultures of lactic streptococci. A trace of phage falling into a vat in which a susceptible streptococcus is growing multiplies rapidly in the whey. In the course of the manufacturing process the whey is drained off, passed through a centrifugal separator, stored in large tanks and finally carted away by farmers for the feeding of pigs. During all these stages—and more especially during passage through the separator—fine droplets of whey containing a high concentration of phage are broadcast in the atmosphere within and around the dairy factory.

(c) Although phages for the lactic streptococci tend to be type-specific they are not strictly so. It was the (at the time) apparent type-specificity of these phages which formed a strong point in the argument against explaining the seemingly spontaneous occurrence of phage by assuming contamination from external sources. Subsequent work has shown that there are phages which can attack as many as four distinct strains of *Strept. cremoris* (Whitehead and Hunter, 1939). It is thus quite conceivable that, in the surroundings of a dairy factory which has been operating for many years, or even throughout

a countryside where whey has been fed to pigs over a long period, there may be traces of a sufficient variety of phages to lead to attack on quite a wide variety of types of streptococci

On the hypothesis which now seems adequate to cover most of the facts, the introduction of a presumed fresh strain of streptococcus or a strain rendered resistant to a phage into the cheese vats in a given factory is equivalent to providing a ' seed bed ' for a phage which may have lain dormant in the surroundings for months or even years. After the first development of the phage in a cheese vat a constantly increasing infection develops day after day until the concentration of an borne phage becomes so high that it is difficult to avoid infection of cultures of the susceptible streptococcus sown with a normal aseptic technique in the factory.

Even when the above points are taken into consideration the occasional very early phage attack on a freshly isolated streptococcal type is somewhat surprising and leads one to wonder whether contamination with pre-existing phage from external sources explains all the phenomena. Nevertheless on the evidence before us we must for the present abandon the hypothesis of a bacterial origin for phage. The ultimate origin or habitat of phage remains obscure, as indeed does the ultimate habitat of the lactic streptococci themselves.

Summary

Air borne bacteriophage in the surroundings of cheese factories is shown to account for many of the sudden appearances of phage in cultures of lactic streptococci used in cheese manufacture. Previously the seemingly spontaneous occurrence of phage was taken as evidence in favour of a bacterial origin. The streptococcal phages are not as strictly type specific as early work seemed to indicate.

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FURTHER OBSERVATIONS ON THE USE OF SYNTHETIC RESIN AS A SUBSTITUTE FOR CANADA BALSAM

PRECIPITATION OF PARAFFIN WAX IN THE MEDIUM AND AN
IMPROVED PLASTICISER

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Some months after our publication on the use of a synthetic resin—the polystyrene, Distrene 80*—as a mountant for microscopical preparations (Kirkpatrick and Lendrum, 1939), we observed the appearance in some of the

* Obtainable from British Resin Products, Ltd., Great Burgh, Epsom, Surrey

histological preparations of tiny refractile bodies. These occurred both as thin needle-like crystals and as small irregularly curved flat plaques; they were quite colourless and the finer ones became visible only on reducing the aperture of the diaphragm. They had not been noticed in mounts of smears or films.

It has now been found that these bodies are the result of mounting sections from a jar of xylol previously used for dewaxing. Slides were immersed in a strong solution of wax in xylol, and from this were mounted in balsam and in polystyrene; control slides were mounted from pure xylol. Within a fortnight the polystyrene mounts from waxy xylol showed abundant bodies, whereas the balsam mounts showed not a trace of precipitation after two months. Films mounted in balsam from a saturated solution of wax in xylol failed to show precipitation after a year. This toleration on the part of balsam may be explained in part by a persistence of the solvent xylol in the mount; it certainly is a fact that balsam mounts even after years are still tacky in the centre. Other writers (Groat, 1939, 1940; Davies, 1939-40; Lillie, 1941), working with Clarite (a cycloparaffin polymer) and with methacrylate polymers, make no mention of this trouble. It is possible that these substances have the high tolerance for wax shown by balsam or it may be that, in the routine processing, other laboratories do not use the same dish of xylol for de-waxing and for final clearing, a form of economy that we practised only with the diagnostic biopsy material and one that is justifiable if balsam be used. The use of fresh xylol before mounting prevents the formation of the bodies in polystyrene mounts.

When these deposits were first noticed we made enquiries as to the possibility of plasticising with substances other than tricresylphosphate. Our suspicion that the tricresylphosphate was the cause of the deposits was only later transferred, as it proved with justice, to the paraffin wax. The use of dibutylphthalate was suggested to us by Dr Tucker (of the Department of Organic Chemistry) and by Mr M. D. Curwen (editor of *Plastics*); apparently this substance is, on theoretical grounds, more suitable than tricresylphosphate for combination with a polystyrene (Curwen, 1940). A mountant called, for brevity, B.P.S., was prepared as follows.

Five c.c. of dibutylphthalate (B.D.H.) are mixed with 35 c.c. of xylol; in this are dissolved 10 g. of Distrene 80. Preparations made with this solution have shown no degradation after 14 months, while the preservation of colour and the refractive index and the lack of retraction are fully as good as in those prepared with the earlier solution. This medium is just as intolerant of paraffin wax as that made up with tricresylphosphate. The new solution has the distinct practical advantage of forming a less tacky surface at the edge of the coverslip and of hardening there as quickly as balsam.

It may be added that the lapse of time since our earlier publication has given further proof that the synthetic resin preserves the colours of bacteriological preparations very much better than balsam. In histological preparations, the more fugitive stains have been well preserved, for example malachite green and acridine red, eosin and methylene blue and other Romanowsky mixtures. Some of the hæmalum and eosin sections, however, have shown fading, a fact that cannot as yet be adequately explained. These were in an uncontrolled series; where parallel balsam and polystyrene series have been prepared, the hæmalum and eosin staining has proved particularly and equally persistent.

The reader interested in polystyrene should consult the paper of Staudinger and Stanley (1938); the subject of plasticisers is dealt with by Clark (1941).

Summary

The use of a synthetic polystyrene resin (Distrone 80) in place of Canada balsam demands the complete removal of paraffin wax from the slide

The use of dibutylphthalate as the plasticiser has certain advantages and seems in no way to diminish the superiority of this medium over Canada balsam, it does not remove the need of dissolving away all paraffin wax

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THE OPTIMAL SPACING OF VACCINE INOCULATIONS

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It has been customary to give the second dose of a prophylactic bacterial vaccine after an interval of 8-10 days, the reason for this being that about this time the antibody production is at its height. A second dose then, it was argued, should produce the maximum effect.

With the addition of an antigen such as tetanus toxoid to a vaccine it becomes necessary for the attainment of optimal antitoxin immunity to space the doses at an increased interval of 4-6 weeks and the question then arises, will this prolongation which is essential for the toxoid prove disadvantageous to the bacterial immunisation?

The vaccine chosen for the elucidation of this point was one made from *S. typhi murium*, that organism being a natural parasite of the mouse, in which animal it was proposed to carry out the experiment. The pure line "E" strain of mice employed was very susceptible to infection with *S. typhi murium*. The culture was a highly virulent strain "480/40" isolated from a fatal case of food poisoning and kindly supplied by Dr Marion Watson, the vaccine prepared from it being an alcohol killed and alcohol-preserved saline suspension of agar growth at 37° C.

Batches of 20 mice received two subcutaneous inoculations, the first containing 200×10^6 organisms and the second double that amount. Two batches were inoculated with the customary interval of one week, two others received their second injection after a period corresponding to that employed when administering tetanus toxoid, namely 4 weeks. One group of the pair receiving closely spaced doses was tested for agglutinins one week after the second inoculation and for active immunity 3 weeks after that injection, while the other group was tested for agglutinins 7 weeks and for

active immunity 9 weeks after the second inoculation. The same arrangement was carried out in the two groups receiving widely spaced doses, the inoculations being performed on such dates that the tests for active immunity by infection with living *S. typhi murium* could all be carried out at the same time.

Only one death from "natural causes" occurred in the course of the ninety days taken by the experiment, though several took place while the mice were being bled from the tail under ether or during the preliminary heating of the animals in the 37°C. incubator to render the flow of blood from the clipped tail more active, excessive exposure to this temperature readily giving rise to heat apoplexy.

The sera were tested for agglutinin content against a formolised suspension of the O variant *S. typhi murium* "Glasgow O" and active immunity by intraperitoneal inoculation with the virulent strain *S. typhi murium* "480/40" used in preparing the vaccine. A minimal dose of 300 organisms was given; but such is the susceptibility of the E strain mouse to *S. typhi murium* infection that even among the inoculated there were no survivors and the effect of immunisation had, as in previous experiments (Schütze, 1939), to be deduced from the prolongation of life resulting from the vaccination.

Table I gives the somatic titres found in the four inoculated groups, the figures for the sexes being entered separately, as earlier work with E mice had demonstrated diverse sex reactions in respect of antibody formation; in the average for the sexes combined, account was taken of their respective numbers. The geometric mean titres were calculated by assigning the numbers 1, 2, 3, etc. to the different dilutions and taking the average of these figures.

TABLE I

O agglutination titres of mice inoculated with two doses of *S. typhi murium* vaccine at different intervals and bled after varying periods

Interval between doses (weeks)	Time of bleeding after 2nd dose (weeks)	No. of mice bled	Sex	Number of mice with O titres of 1 :							Geometric mean of titre
				15	30	60	120	240	480	960	
4	7	10	M			8		1		1	3.6}
4	7	8	F			2	4			2	4.5}
											4.0
1	7	6	M	1	3	2					2.2}
1	7	8	F		1	2	3	1	1		3.9}
											3.17
4	1	10	M			3	2	4	1		4.3}
4	1	8	F				3	3	2		4.9}
											4.6
1	1	9	M	1	5	3					2.2}
1	1	8	F		1	4	1	2			3.5}
											2.81

It can be seen from table I that a significantly higher titre is obtained with a spacing of 4 weeks between the two vaccine doses than with a spacing of 1 week. This result is achieved whether the mice are tested 7 weeks or 1 week after the 2nd dose. That an increase in spacing is not damaging to the production of agglutinins in man has been demonstrated by Maclean and Holt (1940), who found that a prolongation of the interval between two doses of T.A.B. vaccine from 1 to 4 weeks did not alter the degree of response to the antigens of the vaccine. From this table it is also seen that females have a greater capacity for O antibody formation than males,

which is in accordance with former observations with this strain of mice (Gorer and Schutze, 1938)

In table II are given the results of the test for active immunity carried out at varying periods after the second of two closely or widely spaced doses

TABLE II

Active immunity in mice inoculated with two doses of S typhi murium vaccine at different intervals and given after varying periods a test dose of S typhi murium

Interval between doses (weeks)	Interval from 2nd dose to test (weeks)	No. of mice	Sex	Average length of life of mice (days)
1	9	12	M	9.9
1	9	8	F	9.0
1	9	8	M	10.0
1	9	8	F	11.0
1	3	11	M	11.5
1	3	8	F	10.0
1	3	11	M	9.8
1	3	8	F	10.0
Controls		12	M	6.6
"		8	F	6.1

The degree of immunity conferred by two doses of vaccine is shown in table II to be no less when the customary interval of 1 week is altered to one of 1 weeks, in all cases the survival period of the immunised mice as compared with that of the un inoculated controls has been increased to an equal extent, the controls dying after an average of 6 days and the immunised after about 10 days. That there was no sex difference in respect of average length of life was, it may be noted, unexpected, for on a previous occasion the increased survival time of females was quite definite. The strain of *S typhi murium* "6079", used then in a dose of 20 000 organisms, killed non immunised males in an average of 3.6 days but females only after 4.5 days. To re test this point non immunised E mice were inoculated intraperitoneally with large and small doses of the highly virulent strain "480/40" and with a large dose of the less virulent strain 'Uganda', the results are compared in table III.

TABLE III

Susceptibility of male and female E mice to inoculation with two strains of S typhi murium

No. of mice tested	Sex	Dose and strain of S typhi murium	Average length of life (days)
24	M	180 of '480/40'	6.1
19	F	480 of '480/40'	6.4
24	M	120,000 of '480/40'	4.5
20	F	120,000 of '480/40'	4.5
23	M	102,000 of 'Uganda'	6.2
18	F	102,000 of 'Uganda'	9.7

It is apparent from table III that the strain "480/40" is not more lethal for male than for female mice, whether the dose be large or small, whereas the strain "Uganda", like "6079" previously mentioned, demonstrates a marked difference in resistance between the sexes. It would seem therefore that this difference is elicited only by certain strains, but upon what characters this capacity to differentiate depends is unknown.

Discussion

By increasing the spacing between two doses of *S. typhi murium* vaccine from the customary interval of 1 week to one of 4 weeks no alteration in the active immunity of the immunised mice, as judged by their resistance to a subsequent infection with living *S. typhi murium*, was noticeable. Such a change in the technique of immunisation as is necessitated by the introduction of tetanus toxoid into a typhoid-paratyphoid vaccine, for example, should not prove disadvantageous in the immunisation of man. Indeed, if the antibody titre of an inoculated animal is an index of the immunity achieved, though this is by no means sure, increased spacing must be regarded as an improvement in the method of inoculation, for the 31 mice receiving the second dose one week after the first had a mean O titre of 1:60, while the 36 mice whose second inoculation fell four weeks after the first attained a mean titre of 1:160.

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576. 851. 2 (*Diplococcus mucosus* von Lingelsheim)

DIPLOCOCCUS MUCOSUS VON LINGELSHEIM: A DESCRIPTION OF A STRAIN WITH COMMENTS ON THE SYSTEMATIC POSITION OF THE ORGANISM

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Capsulated Gram-negative diplococci were isolated by von Lingelsheim (1906, 1908) from the nasopharynx and cerebrospinal fluid and called by him *Diplococcus mucosus*. Elser and Huntoon (1909) isolated similar organisms from the nasopharynx. Wilson (1928) and Wilson and Smith (1928) did not encounter capsulated strains in their study of nasopharyngeal *Neisseriæ*, but suggested that such strains might be mucoid variants of *N. pharyngis*. Cowan (1938) described two strains of capsulated *Neisseriæ* isolated from the cerebrospinal fluid of patients after operation for cerebral tumour and suggested that *Diplococcus mucosus* should be called *Neisseria mucosa*. In view of these differing opinions, and of the rarity of capsulated *Neisseriæ*, the following observations are reported on a strain isolated from the cerebrospinal fluid of a case of meningitis.

SOURCE OF THE STRAIN

The strain was isolated by Major J. Boycott, R A M C, from a man aged 22 years, who was seen on account of bed wetting and general slovenliness and who was found to have scanning speech and intention tremor. Pyrexia and signs of meningitis supervened and lumbar puncture yielded a purulent fluid with Gram negative diplococci in the films, some of them intracellular. Sulphapyridine treatment was instituted and the patient improved rapidly and made a complete recovery.

CHARACTERS OF THE STRAIN

It was noted by Major Boycott that the growth in culture was more rapid and profuse than that obtained with meningococci, and that in films the cocci showed less variation in size and depth of staining than is usual with that organism. Glucose was fermented and maltose slightly fermented. There was no agglutination with meningococcal serum.

On plating out the strain two types of colony were obtained. Type A was opaque and round and had a regular edge, glossy surface and highly convex elevation. Type B was translucent and round and had an irregular edge, wrinkled surface and flat conical elevation. This colonial differentiation persisted during repeated subcultures over three months. Neither type showed variant colonies resembling the other.

Morphology

On all media both types showed Gram negative cocci in pairs, rarely in tetrads, and occasionally (more frequently in type A) in large irregular clusters. They were spherical, with slight flattening of the opposed surfaces, 1μ in diameter and non motile. Capsules were demonstrated by nigrosin and by Muir's method on several occasions during the first three weeks of study. After three months in culture no capsules were demonstrable.

Cultural characters

Incubation was at 37°C unless otherwise stated.

Agar plate **Type A** *24 hours* Good growth, confluent and mucoid along initial stroke. Separate colonies ($1.5-2\text{ mm}$) round, with regular edge, domed, glossy, opaque, yellow grey, viscous and easily emulsified. *48 hours* Colonies slightly increased in size, compact, high convex and mucoid. *4 days* Colonies $3-5\text{ mm}$, radially striated, with convex centre and bevelled edge.

Type B *24 hours* Good growth, confluent along initial stroke. Separate colonies up to 2 mm , low conical with slightly irregular edge and matt surface, translucent, blue grey by transmitted light, soft and easily emulsified. *48 hours* Colonies up to 3 mm , with flattened border, concentric contouring and dry, slightly wrinkled surface. *4 days* Colonies up to 4 mm , low conical with flat periphery, irregular edge and wrinkled surface.

Horse blood agar plate Growth equal to that on plain agar. Similar differentiation of A and B types of colony. Ill defined clearing but no definite haemolysis under growth of type A where confluent. No change in blood with type B.

MacConkey's agar plate *24 hours* Both types showed umbonate colonies, smaller than on plain agar and less clearly differentiated. *48 hours* **Type A** Domed, mucoid, opaque colonies. **Type B** Flat, translucent, wrinkled colonies. Differentiation now marked.

Loeffler's serum slope. 24 hours. **Type A.** Confluent, yellow-white mucoid growth with glossy surface. **Type B.** Almost confluent growth of small grey colonies with dull surface. No liquefaction with either type after seven days.

Heart broth. 24 hours. **Type A.** Marked turbidity. **Type B.** Slight turbidity. Both types produced a slight granular deposit which was easily shaken up. 48 hours. Both types showed increased turbidity and type A a surface ring.

MacConkey's broth. 24 hours. Both types caused turbidity but produced no acid.

Gelatin stab. At room temperature both types gave growth down the needle track and spread slightly on the surface. No liquefaction after four weeks.

Biochemical reactions

In peptone water sugars type A produced acid in glucose but not in maltose, mannitol, lactose, sucrose or salicin. Type B fermented none of these substrates. Tubes were incubated for three weeks and good growth was obtained. On litmus serum agar slopes type A produced acid in glucose but not from maltose nor sucrose. Type B produced no acid in these sugars. With both types litmus milk was unchanged and both were methyl red—, Voges-Proskauer—, indole—, catalase+, H_2S trace.

Metabolic behaviour. No growth occurred anaerobically. At room temperature growth was slower than at 37° C., with marked differentiation of types A and B.

Serology

No agglutination with group 1 or group 2 meningococcal serum.

Animal inoculation

One-half ml. of a 24-hour broth culture of each type was inoculated into two mice intraperitoneally and one mouse subcutaneously. None of the mice showed any lesion when killed seven days after inoculation, and heart blood and spleen were sterile.

DISCUSSION

The demonstration that this strain of *Neisseria* was capsulated suggested that it was an example of *Diplococcus mucosus*. The capsules as observed with nigrosin were large and definite and unlike the indefinite haloes demonstrable round meningococci. In addition, they were stained by Muir's method, so that they appear to be definite structures. Using the word capsule in that sense, it is still possible to subscribe to the statement of Elser and Huntoon that *Diplococcus mucosus* is the only capsulated member of the genus *Neisseria*. They noted as its distinguishing features capsulation, growth on plain agar, growth at room temperature and succulent mucinous colonies. The A type here described fulfils these criteria. The B type appears to be a rough variant with colonies resembling those of *N. pharyngis sicca*. Rake (1933) isolated strains of meningococci and of *N. flavescens* which exhibited similar colonial variation. Wilson and Smith found great variation in colony form among strains of *N. pharyngis* and in the same strain at different times and on different media. It is clear that colonial variation is not a valid criterion for species differentiation in the *Neisseriæ*, and the observations recorded here show that capsulated strains vary in colonial form as do the non-capsulated varieties.

Cowan's two strains of *Diplococcus mucosus* fulfilled Elser and Huntoon's criteria, but differed in several respects from the strain described here.

They produced acid in glucose and after twenty one days from lactose, while this type A produced acid in glucose only and type B in none of the sugars tested. His strains gave acid and clot in litmus milk, with this strain litmus milk was unchanged. Here again Wilson and Smith found great irregularity in the fermentation reactions of *N. pharyngis* and in this respect also *Diplococcus mucosus* falls into line with that group. Cowan's strains liquefied gelatin after seven days. This strain did not do so after four weeks, which agrees with von Lingelsheim's description, although he does not state how long his strains were observed.

The ability of this strain and of Cowan's strains to grow on MacConkey's agar merits attention. Three recently isolated strains of *N. pharyngis* did not grow on it, but further tests are needed before the significance of this observation can be assessed.

Cowan's strains in doses of 0.05 ml. of broth culture killed mice regularly, and von Lingelsheim found that his strains were pathogenic for mice. Evidently some capsulated *Neisseria* are pathogenic, but this strain resembles the majority of *Neisseria* in being non pathogenic for mice.

The evidence in favour of the pathogenicity of *Diplococcus mucosus* for man is inconclusive. One of von Lingelsheim's strains came from the cerebro-spinal fluid of a patient with pneumonia and meningeal symptoms. Cowan's strains were isolated from the cerebro-spinal fluid of cases of infection following operation for brain tumour, but there was no histological evidence of inflammation in the one which came to post mortem. The strain here described was isolated in pure culture from a case of meningitis but on one occasion only. It seems highly probable that it was the causative organism, but Elber and Hinton conclude that various *Neisseria* isolated by von Lingelsheim in similar circumstances were contaminants and advise caution in claiming pathogenicity for such strains. Even if the pathogenicity of *Diplococcus mucosus* for man were established that would not differentiate it from the *N. pharyngis* group, for Garland (1923) reports a case of meningitis due to *N. catarrhalis* and cites three others, and Branham (1930) reports an outbreak due to *N. flaucescens*. Thus nasopharyngeal *Neisseria* other than the meningococcus do occasionally cause meningitis in man.

The observations recorded in this paper show that strains of capsulated *Neisseria* which are apparently examples of *Diplococcus mucosus* vary in cultural and biochemical characters. The differences between the strain here described and others previously described remove colonial characters, fermentation reactions and pathogenicity for mice from the list of differential characters of the organism. It appears that capsulated *Neisseria* which grow on plain agar at room temperature have many resemblances to the *N. pharyngis* group. Further study of such strains, including serological investigation, is required before deciding to constitute a species *N. mucosa* as proposed by Cowan, and it seems possible that such studies will show the strains to be related to the *N. pharyngis* group as Wilson and Smith suggest.

SUMMARY

A strain of *Diplococcus mucosus* is described. It differs from strains previously described in having a rough variant colony, in its fermentation reactions and in its lack of pathogenicity for mice. These differences favour the suggestion that the organism is a variant of the *Neisseria pharyngis* group. The systematic position of the organism is uncertain and study of further strains is required.

I desire to thank Major Boycott for sending the strain and for notes about the case from which he isolated it.

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BOOK RECEIVED

The biologic fundamentals of radiation therapy

By F. ELLINGER. New York : Elsevier Publishing Co., Inc. , London : H. K. Lewis and Co. Pp. x and 360 , 79 text figs. 30s.

This short book is a translation of one published in German in 1935 and since brought up to date by the author. In it is presented a survey of an immense literature on the biological effects of radiation.

About one third of the book concerns the effects of light and light therapy and includes the author's own contributions. The remainder deals largely with the effects of X and gamma radiation on organs and tissues, including an account of the well known theories of radiation action. A small section deals with the effects of radiation in malignant disease. The chapters on the effects on the skin both of ultraviolet light and of X-radiation are the most comprehensive and indicate the author's own great interest in this field. The effects of light on the skin are excellently summarised and the description of the underlying mechanisms involved certainly provides a biological basis for an understanding of the problems associated with light therapy. The chapter on skin effects following roentgen rays is also well written ; if a criticism may be levelled here it is of the author's assumption that skin erythema following radiation therapy should stop short of the stage of moist desquamation. This stage, which is described among the irreversible skin changes, is regarded by the author as a burn and as resulting from "carelessness or faulty technique", whereas a skin reaction which reaches moist desquamation is deliberately envisaged in many cases as the necessary accompaniment of a curative dose of radiation. Such a reaction, while leaving no permanent visible mark on the skin, is irreversible in that such skin is less able to tolerate subsequent radiation.

In a short book such as this it is impossible for the author to deal adequately with all the biological aspects of radiation and the physical side of radiation therapy has been largely omitted. From some stand-points this is a pity, since it is the author's intention to provide the armamentarium from which therapeutic measures can be evolved and these depend fundamentally on the measurement of dosage. In respect of gamma rays particularly the unit of dosage in roentgens is mentioned only as a footnote, although the gamma roentgen has been accepted internationally since 1937.

While the book will be found of interest by many it will appeal primarily to research workers in the many fields of radiation as providing a well documented digest of the literature. There is a bibliography of 1100 references.

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